INVESTIGATIONS INTO THE FIBRINOLYTIC SYSTEM OF HUMAN MIXED NATIVE SALIVA

by

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CONTENTS

Volume I

ABSTRACT

INVESTIGATIONS INTO THE FIBRINOLYTIC SYSTEM OF HUMAN MIXED NATIVE SALIVA

Fibrinolysis is defined and a review of modern and historical concepts of the fibrinolytic system is presented.

The nature of human mixed native saliva (MNS) and the problems of its investigation are discussed. The literature concerning the fibrinolytic activity of MNS, parotid and submandibular/sublingual saliva is reviewed and the lack of agreement noted.

Preliminary investigations are described which establish the sensitivity of the fibrin plate technique and the consistent presence of specific plasminogen activator activity in MNS. Considerable inter and intra-personal variation in this activity is observed, but no variation due to sex is recorded. The stability of plasminogen activator is assessed at various temperatures over varying lengths of time.

Examination of supernatants and filtrates of MNS are described as well as of the isolated parotid and submandibular/sublingual salivas. Evidence is presented suggesting the absence of a soluble activator and this is later substantiated in density gradient and gel chromatography experiments.

The elimination of a soluble activator establishes the activator activity as residing in the salivary pellet which consists of salivary mucus, bacteria, leukocytes and desquamated epithelial cells. Systematic investigations using fibrin plates and fibrinolytic autographs to test both fresh and freeze-thawed suspensions of cells in tris buffer and MNS supernatant establish the desquamated epithelial cells and cell fragments/

fragments as providing by far the greatest amount of plasminogen activator activity. More specific investigations of the salivary flora are described.

The streptokinase-activated proactivator activity of MNS is investigated and two components are separated, a soluble proactivator and a cell-associated proactivator.

Using the techniques of two dimensional immunodiffusion, tanned red cell haemagglutination inhibition immunoassay, and by observing the molecular weight and action of fractions of MNS supernatant obtained by gel chromatography (Sephadex G200) when combined with streptokinase and incubated upon bovine fibrin plates, it is established that all the streptokinase-activated soluble proactivator can be accounted for by the presence of plasminogen.

Systematic examination of components of the salivary pellet using bovine fibrin plates and bovine fibrin autographs in the presence of streptokinase both with and without epsilon-aminocaproic acid is described and although all cell types examined display proactivator activity, the most prominent are epithelial cells. The possibility that some of this proactivator activity might also be due to plasminogen is discussed.

It is concluded that no soluble plasminogen activator exists in human saliva and that the activator activity of MNS is brought about by the incubation of epithelial cells possessing weak activator activity with plasminogen secreted in the saliva of the major salivary glands. An experiment demonstrating this is described. A diagramatic representation of the fibrinolytic system (activator and proactivator) in human MNS is presented.

A/

A short discussion as to the possible function of the fibrinolytic system in MNS and an indication of further research topics ends the Thesis.

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PART I

INTRODUCTION

Chapter 1 (i) Fibrinolysis.

(ii) Historical and Modern Concepts of Fibrinolysis.

A Review.

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CHAPTER I

(i) FIBRINOLYSIS

"Fibrinolysis can be defined as the enzymatic breakdown of fibrin to fragments which are no longer able to form a coherent net" (von Kaulla, 1963). Although the enzymatic breakdown can be brought about by a variety of proteolytic enzymes, fibrinolysis in the 'clinical sense' is produced by the enzyme plasmin, the proteolytic action of which is non-specific but in the presence of inhibitors in the blood, attacks only fibrin to which it is adsorbed (Ratnoff, 1953). Dissolution by the fibrinolytic system is determined not only by the presence of plasmin, but also and primarily, by its content of substances capable of converting plasminogen to plasmin, plasminogen activators (Alkjaersig et al, 1958a).

(ii) HISTORICAL AND MODERN CONCEPTS OF FIBRINOLYSIS: A REVIEW

In his work 'De Sedibus et Causis Morborum' (The Seats and Causes of Diseases), Morgagni (1769) observed that blood remains fluid in cases of sudden death. John Hunter (1794) made the same observation while hunting, but it was not for many years that properly controlled scientific investigations into the relationship that exists between exercise and this phenomenon were made (Fearnley and Lackner, 1955: Billimoria et al, 1959: Ogston and Fullerton, 1961: Menon et al, 1967 and Cash, 1968 and 1969).

Denis (1838) recorded that fibrin can dissolve spontaneously in an alkaline solution of 16% nitrate. Liebig (cited by von Kaulla, 1963) was at first unable to confirm this but he did so later when, like Denis, he used human fibrin in place of bovine fibrin he had used previously. Thus, from the earliest days, a species difference was recorded which has since been largely ignored.

Plosz (1873) noted that fibrin left in salt solution dissolved, but that the ability to dissolve was eliminated by repeated salt extraction. From this, he concluded that the extraction must elute a material essential for the dissolution of the fibrin. Green (1887) noted that the rate of dissolution is much less rapid in serum than in salt solution and Denys and Marbaix (1889) found that a thermolabile proteolytic agent appe ars in serum after mixture with chloroform and ether. Delezenne and Pozerski (1903) showed that this proteolytic agent in the serum has the ability to digest gelatine and casein: an activity that is inhibited by the addition of untreated serum.

The word 'fibrinolysis' was first used by Dastre in 1893. He studied the influence of repeated haemorrhage upon the fibrinolytic activity of the/

the blood of dogs and noted that fibrin dissolves when left in contact with the blood from which it is derived. This dissolution he called 'fibrinolysis'.

Nolf (1904 and 1905) published his work on the physio-pathology of fibrinolysis, based on the principle that peptone injections produce incoagulability of the blood in hepatectomized dogs (Gley & Le Bas, 1897). Nolf reported that this phenomenon is associated with a marked increase in fibrinolytic activity that is due to irritation by peptone of the vascular wall and the consequent release into the circulatory system of a substance that enhances normal fibrinolysis. He found it much easier to induce a fibrinolytic reaction by peptone in dogs which are overfed on meat than in dogs not fed meat, thus observing a possible association between diet and the fibrinolytic activity of blood. In 1906, Morawitz offered a partial explanation for Hunter's observations upon chased animals by noting that the blood from victims of sudden death contains no fibrinogen and speculated that this results from its digestion by an enzyme. Furthermore, the victim's blood can destroy the fibrinogen and fibrin of normal blood. Hedin (1903) found spontaneous fibrinolytic activity in the globulin fraction of ox blood and Opie & Barker (1907) were able to repeat this work and, in addition, show that fibrinolytic activity induced by chloroform and ether treatment of serum, resides in the globulin fraction of plasma obtained by salt precipitation. Apart from some work by Nolf (1921) which showed that proteolytic activity accompanied by fibrinolysis can be generated in mammalian and avian plasma by chloroform, little other work of significance took place until 1933.

Streptococcal fibrinolysis was discovered at the Rockefeller Institute in 1933 by Tillett and Garner. It is from this very important contribution to the knowledge of fibrinolytic mechanisms that most subsequent work on fibrinolysis stems, and thus it forms the natural watershed between the largely fragmentary studies and the systematic and/

and interrelated work that has continued ever since.

Tillett and Garner (1933) found that the filtrates and broths of beta-haemolytic streptococci cause rapid liquifaction of human blood clots. Milstone (1941) showed that fibrin obtained from highly purified fibrinogen is resistant to the lytic action of streptococcal filtrates, but that lysis occurs when a small quantity of euglobulin fraction is added to the test system. He called this accessory component the 'lytic factor'. Christensen and MacLeod (1945) demonstrated that beta-haemolytic streptococci produce an activator or kinase which they termed 'streptokinase'. Streptokinase converts an inactive precursor which they called 'plasminogen' to 'plasmin', the active proteolytic enzyme. Milstone's lytic factor is thus seen to be plasminogen, and its active form plasmin can also develop autocatalytically from plasminogen when serum is treated with chloroform. It was now apparent why highly purified fibrin is not lysed by streptococcal filtrates since purification diminishes the contaminating plasminogen and there is consequently little enzyme precursor for streptokinase to convert to plasmin.

The more important relevant aspects of the fibrinolytic system will now be discussed and the Historical Review continued but under separate headings.

PLASMINOGEN

Plasminogen, a beta globulin (Robbins and Summaria, 1963) is the inactive precursor of the enzyme plasmin and is present in the blood and most body fluids (Sherry, 1966). The presence of plasminogen in various human tissues was suggested by Kowalski et al (1958) and Wille (1957) demonstrated appreciable amounts of plasminogen and antiplasmin in the human placenta. In various exudates and transudates, a correlation exists between plasminogen and fibrinogen levels: where fibrinogen is low or absent, plasminogen is reduced or absent and where fibrinogen is abundant, plasminogen is present in high concentrations (Sherry, 1966). Nitta et al, (1967) suggest plasminogen might be present in saliva. No quantitative estimation has, however, been made nor has its presence been determined by direct methods. The site of production of plasminogen is uncertain. Barnhart and Riddle (1963) using a fluorescent antibody-antigen technique have shown that plasminogen is present in, and therefore could be produced by, eosinophils. This work does not seem to have been repeated.

Sgouris et al (1960) estimated the plasminogen levels in fresh human plasma with a caseinolytic method and a mean value of 2.58 (1.72 - 3.20) caseinolytic units per millilitre was determined. Plasmin activity has also been assessed by its action upon modified fibrin plates, its esterase activity with synthetic esters and by clot lysis (Troll et al, 1954: Alkjaersig et al, 1959 and Wolf, 1968). An elegant and more sensitive method for the estimation of plasminogen was devised by Ludlam and Das (1971) who adapted the tanned red cell haemagglutination inhibition immunoassay (TRCHII) for plasminogen.

Characterization:

Milstone first precipitated plasminogen out in the euglobulin fraction of/

of plasma in 1941. Plasminogen extraction from Cohn Fraction III with a strong mineral acid (Christensen and Smith, 1950) was modified by Kline (1953) and Kline and Fishman (1961) to obtain a higher degree of purity. Concern about the technique however was expressed by Slotta and Michl (1962) who suggested acid treatment procedures probably produce several changes in the plasminogen molecule including changes in molecular weight and substrate specificity. This objection was overcome when Wallen and Bergstrom (1959) and Alkjaersig (1964) prepared plasminogen completely soluble at neutral pH by chromatographic techniques. Wallen and Wiman (1972) have demonstrated ten different forms of plasminogen using zymographic analysis after starch gel electrophoresis. However, of these, only six have electrophoretic properties identical to those in human plasma. The other four forms they suggest are probably altered forms of plasminogen brought into being by proteolytic degradation during the extraction processes.

Wallen and Wiman (1970, 1972) have identified two groups of plasminogen from Cohn Fraction III, namely Glutamic acid NH_2-terminal plasminogen (Glu-plasminogen) and Lysine (or valine) amino terminal residue plasminogen (Lys-plasminogen).

The chief characteristics of these two forms have been summarised by Collen and Maeyer (1975).

Glu-plasminogen

1. NH_2-terminal glutamic acid.
2. Isoelectric points pH 6.0 and 6.6.
3. Electrophoretic mobility on starch gel very similar to that of plasminogen in unfractioned plasma.

Lys-plasminogen

1. NH_2-terminal lysine (or valine).
2. Isoelectric points pH 7.3 and 8.8.
3. Different electrophoretic mobility from/

from that of plasminogen in unfractioned plasma.

Summaria et al (1973)have suggested that both forms are present in circulating human plasma but the work of Collen and Maeyer (1975) and Collen et al (1975) supports the view of Wallen and Wiman (1972) that the Glu-plasminogen is the native form and that Lys-plasminogen represents proteolytic degradation products of the native form. Collen et al (1975) obtained Glu-plasminogen from fresh frozen human plasma with over 90% recovery by affinity chromatography with lysine-coupled agarose in the presence of the plasmin inhibitor aprotinin. They also showed that the highly purified material had 20 - 24 CTA units/mg. protein; an apparent molecular weight of 90,000 $\pm$ 2800 and that its six main molecular forms had isoelectric points ranging from pH 6.1 - 7.1. Plasminogen similarly prepared from Cohn Fraction III paste pre-treated with aprotinin had the same molecular forms and, in addition, other isoenzymes with isoelectric points ranging from pH 6.5 - 8.4 with an amino-terminal lysine residue and an apparent molecular weight of 84,300 $\pm$ 3,100 were present.

Using radioiodine - labelled plasminogen preparation, Collen and Maeyer (1975) showed that when these preparations were injected into men, the half life of Glu-plasminogen was 2 - 2.5 days while that of Lys-plasminogen was only 0.8 days.

These data indicate that plasminogen isoenzymes with high molecular weight, low isoelectric points, glutamic acid amino-terminal residues and a long half life represent the native circulating plasminogen.

PLASMIN

The conversion of plasminogen to its activated form plasmin is a proteolytic process and according to Alkjaersig et al (1958b) can be considered analogous to the formation of other proteolytic enzymes from/

from their inactive precursors.

Robbins et al (1967) using Lys-plasminogen found that the polypeptide chain of plasminogen is cleaved by urokinase at a single arginyl-valine bond with apparently no molecular weight loss, and that the active plasmin molecule produced is composed of two polypeptide chains of different sizes linked by at least one disulphide bridge.

The molecular weight of the smaller chain (light chain) is about 25,700 (Summaria et al, 1967) and that of the larger fragment (heavy chain) 48,000 (Summaria and Robbins, 1971).

However, during activation, there is a weight loss of plasminogen from 81,000 - 75,400 (Barlow et al, 1969). The difference in molecular weights would be explainable by the liberation of a peptide fragment but the search for such a material has been unsuccessful. Recent work by Wiman and Wallen (1975) suggests a two stage mechanism. In the first stage, an 8,000 molecular weight peptide is released from the NH_2-terminal part of Glu-plasminogen. The remaining protein represents one intermediate which in the second stage is cleaved to give the plasmin molecule consisting of a light and heavy chain which are connected by at least one disulphide bridge. The appearance of enzymatic activity is related to the formation of the two chain plasmin molecule. The intermediate product seems to be enzymatically inactive. Wiman (1973) reported that the material released in the first step of the activation consisted of two peptides designated peptide I and peptide II. The work of Rickli and Otavsky (1973) points in the same direction and recently Rickli (1975) has prepared a model of the activation mechanism. (Diag. 1.).

Step 1.

Proteolytic attack of urokinase in the NH_2 terminal region of Glu-plasminogen which leads to the liberation of a protein moiety and the concomitant formation of a protein component still consisting of a single polypeptide/

polypeptide chain. This protein is enzymatically inactive and it represents an intermediate activation product.

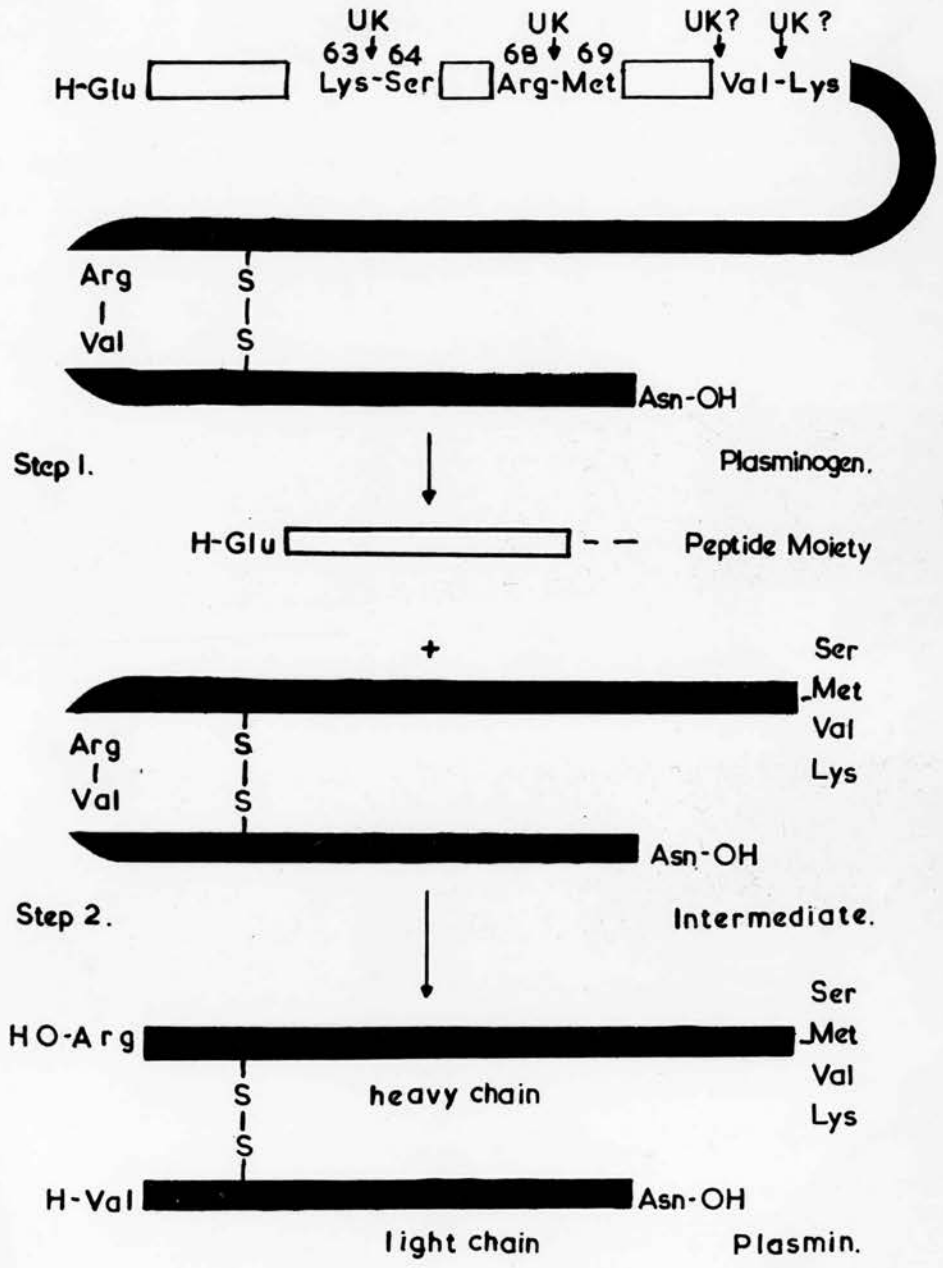
Step 2.

The intermediate protein is hydrolysed by urokinase at a single peptide bond apparently without liberating peptide material, thus forming the active plasmin molecule. The enzyme is consistently described as containing two polypeptide chains of different size, a heavy and a light, chain which are connected by at least one disulphide bond. The experimental evidence indicates that the bond cleaved in the second stage is an arginyl-valine bond as originally observed by Robbins et al. (1967). According to these authors, the active centre is located in the light chain.

With catalytic amounts of streptokinase, the nature and sequence of events are probably the same as with urokinase and it appears that similar and even identical activation products are obtained. With stoichiometric amounts of streptokinase, however, the order of cleavage of peptide bonds is reversed. Nevertheless, it appears this pathway finally yields activation products consistent with those described above. (Rickli, 1975).

Plasmin itself is non-specific and can digest many proteins including fibrinogen and fibrin. It is in the presence of inhibitors, e.g. in plasma, that the relative substrate specificity for fibrin is conferred (Ratnoff, 1953).

Diagram 1.



Schematic representation of the two-step activation mechanism of human plasminogen induced by urokinase (UK). The arrows indicate the observed cleavage of peptide bonds. (Rickli, 1975).

STREPTOKINASE AND PROACTIVATOR

The early work upon streptokinase culminated with Christensen and MacLeod (1945) demonstrating that beta-haemolytic streptococci produce an activator capable of converting plasminogen to plasmin. Mullertz and Lassen (1953) reported that the activation of plasminogen by streptokinase is indirect and requires a further factor which they called proactivator.

When streptokinase is placed on a bovine fibrin plate no lysis develops. When streptokinase is placed on a human fibrin plate , a zone of lysis does develop. Both plates contain plasminogen , therefore , either a species difference manifest in a variant of the plasminogen molecule exists which is not susceptible to activation by streptokinase, or an additional 'factor' exists in the human plate upon which streptokinase first acts and which in turn , when activated , activates plasminogen (Mullertz & Lassen , 1953 : Mullertz , 1955 & 1956).

If proactivator exists (the 'factor' above) , it is probable that a two stage reaction is involved. Streptokinase initially would react rapidly and stoichiometrically with proactivator in plasma (Troll & Sherry, 1955) to form activator which would convert plasminogen to plasmin (Alkjaersig et al , 1958b). The existence of proactivator as a specific substance is questionable. An early alternative , quoted by Amery and Claeys (1970) but without citation , is termed the inhibitor theory. This theory presupposes the presence of large amounts of streptokinase inhibitor in the blood of most species, except man and monkey. This is unlikely since only a small amount of activator is produced in any resistant species, even when large amounts of streptokinase are used and the addition of a small amount of human euglobulin is capable of promoting fibrinolysis (Kline, 1960).

De Renzo (1960) observed that proactivator had never been isolated and human plasminogen had never been prepared devoid of proactivator/

vator activity and, consequently agreed with Ablondi and Hagan (1956) that plasminogen and proactivator are probably one and the same thing. Little definitive work seems to have been done at this time to elucidate the chemical sequence of events in the interaction of streptokinase and plasminogen and virtually nothing was known about the molecular nature of the products found in the interaction; probably because the generation of activator, activation of plasminogen and autodigestion of the enzymes are occurring simultaneously. However, it was known that the relative concentrations of human plasmin and bovine plasminogen activator demonstrable in a streptokinase - human plasminogen reaction mixture depends primarily upon the ratio of streptokinase and plasminogen. When streptokinase is present in high concentrations relative to plasminogen, activator activity predominates, whereas an excess of human plasminogen favours the generation of plasmin activity (Kline and Fishman, 1961: Markus and Ambrus, 1960: De Renzo, 1960 and Alkjaersig et al, 1958b). The work of Kline and Fishman (1961)has confirmed the results of Zylber et al (1959) that streptokinase reacts with plasmin to form activator activity and that such a complex represented one molecular form of activator. Davies et al.(1964) found the molecular weight of the streptokinase -plasminogen reaction product very close to the sum of the molecular weights of unmodified streptokinase and unmodified plasminogen, but he conceded in his discussion upon this finding that the reaction product could well be a streptokinase-plasmin product as had been suggested by Zylber et al.(1959) and Alkjaersig et al (1958b) as the molecular weight of this reaction product would be very close to that of a streptokinase-plasminogen product if such a product existed. Thus in respect of streptokinase, 'activator' was believed to be a complex of human plasminogen or plasmin and streptokinase (De Renzo et al, 1967: Summaria et al, 1969: Ling et al, 1965 and Hummel et al, 1966), and that human plasminogen can be equated with Mullertz's proactivator.

In/

In 1971, Kline and Ts'ao demonstrated that spontaneous plasmin activity can be removed almost completely from human plasminogen preparations by extraction with soybean trypsin inhibitor and treatment with diisopropyl fluorophosphate. The conversion of this plasminogen to plasmin by catalytic amounts of streptokinase then proceeds independently of the availability of preformed plasmin. Thus there is the added possibility of streptokinase being capable of activating plasminogen directly without the prior formation of a plasmin complex or any other plasminogen activator.

Reddy and Markus (1972) agree that streptokinase can activate human plasminogen directly in the absence of spontaneous plasmin, but present evidence which they claim shows that streptokinase does not activate human plasminogen by a direct catalytic process, but that the streptokinase-plasminogen complex is the predominant activator species and that plasmin when present can act as proactivator. Putting the problem in perspective, Reddy and Markus (1972) feel there is sufficient evidence to show that when streptokinase is added to human plasminogen, it immediately forms stable stoichiometric complexes with plasmin and plasminogen, both of which can function as activators, leaving little, if any, streptokinase in the free state. Thus there is no need to ascribe to streptokinase an independent enzymatic role, albeit that in highly artificial circumstances, it may be capable of activating bovine plasminogen. The question arises as to how this happens. The data presented by Reddy and Markus (1972) and independently by McClintock and Bell (1971) provides evidence that an active centre is formed in the zymogen molecule upon interaction with streptokinase. Further evidence for an active site was provided by Reddy and Markus (1973). In addition, they were also able to show that pancreatic trypsin inhibitor abolishes both the activator activity and the ability to react with an active centre - specific reagent. This is accomplished, not by displacement of streptokinase, but by the formation of a ternary complex with streptokinase-plasminogen.

Recently, /

Recently, evidence has been presented by McClintock et al (1974) for the existence of two distinct pathways of streptokinase - mediated activation of human plasminogen. They have observed with catalytic amounts of streptokinase the cleavage of Glu-plasminogen first into fragments with a molecular weight of 80,000 and 5,000 followed by a cleavage of the 80,000 fragment into 60,000 and 20,000 fragments. According to NH_2 terminal analyses the small peptide occupies the NH_2 terminal part of Glu-plasminogen. The fragments with a molecular weight of 60,000 and 20,000 possess NH_2-terminal lysine and valine respectively and may, therefore, correspond to the heavy and light chains of plasmin generated by urokinase.

Interaction of Glu-plasminogen with stoichiometric amounts of streptokinase leads to the formation of a plasminogen-streptokinase complex.

The peptide bond cleavage of plasminogen within this complex occurs in the reverse order to that of the activation with catalytic amounts of streptokinase. A first split produces fragments with molecular weights of 65,000 and 20,000 and from the heavier component a 5,000 molecular weight peptide is then cleaved to yield a remaining fragment with a molecular weight of 60,000.

NH_2-terminal analyses indicate however, that the nature of the peptide bond, cleaved in both pathways, is most likely the same. The reason for the difference in sequence of events must apparently be sought in conformational alterations in the plasminogen molecule. It is assumed that such changes or rearrangements in the zymogen occur when it combines with streptokinase to form the stoichiometric complex. Thus the concept of proactivator as Mullertz understood it, a separate substance in human plasma and in no way to be confused with plasminogen and essential for streptokinase bovine plasminogen activation might reasonably be thought to be unnecessary for an understanding of the mode of action of streptokinase. However, evidence is/

is accumulating that, (1) some streptokinase activated plasminogen proactivator does after all exist and (2) a plasminogen proactivator which is not activatable by streptokinase also exists.

1. Takada et al (1965, 1968, 1969 and 1969) first repeated the work of Mullertz and Lassen (1953), then after using the heated plate technique, claimed that proactivator cannot be plasminogen or plasmin because the plate is not lysed even after the proactivator is activated by streptokinase. Accordingly, they claim proactivator cannot simply be a complex of streptokinase and plasminogen.

A heated fibrin plate is not simply a plasminogen free plate as the heating changes the reactivity of the fibrin (Shiba et al, 1973) and therefore, on this evidence, plasminogen cannot be excluded from the role of proactivator.

However, Takada et al.(1970) claim to have fractioned by gel filtration, a proactivator (called proactivator A) for both human and bovine plasminogen and have shown that there is no cross reactivity of this substance with highly purified preparations of plasminogen in immunodiffusion tests. Furthermore, a mixture of streptokinase and proactivator could not digest casein in the absence of plasminogen. Small amounts of streptokinase converted this isolated proactivator into activator which in turn converted human plasminogen to plasmin, but the same amount of streptokinase could scarcely activate plasminogen in the absence of proactivator. Takada was able to further show that this isolated proactivator is acid labile, and loses its activity even at 56^{o}C whereas plasminogen is only denatured over 85^{o}C. He quotes Lassen (1952) as his authority for this claim of plasminogen stability, but Lassen in fact made no such claim. If his graphs are studied, it will be seen that denaturation is largely a function of time as well as temperature. Lassen effectively destroyed all available plasminogen in a plate in fifteen minutes at a temperature of 95^{o}C and in fifty-five minutes at 70^{o}C.

At/

At 60°C, only about 20% of undenatured plasminogen remained after sixty minutes. Lassen did not continue heating beyond this time and clearly Takada's claim in reference to Lassen's work is suspect. Takada concluded that his findings were in no way incompatible with the finding that streptokinase forms a complex with human plasminogen or plasmin (indeed he refers to human plasminogen as 'proactivator B') nor even that it attacks human plasminogen directly, but simply that that was not the whole story. Further characterization of 'proactivator A' (Takada et al, 1972) has led to an estimation of its molecular weight, 275,000 ± 25,000. Proactivator A migrates in the $alpha_2$ macroglobulin region in electrophoresis, is very homogeneous and has a sedimentation rate of 11.7S. This isolated substance was once more shown to promote caseinolysis induced by human plasminogen and streptokinase, but had no effect on the activation of human plasminogen by urokinase.

The fact first established by Blatt et al (1964) and confirmed by Reddy and Markus (1972)in their experiments that maximum activity towards human plasminogen is obtained when streptokinase and plasminogen are present in a 1:1 molar ratio limits, Reddy claims, the choice of proactivator to either plasminogen or plasmin. Clearly, if any other proactivator, such as proactivator A had been responsible for the mechanism of activation, the streptokinase-plasminogen ratio at maximum activator activity would have been less than 1.0. Not an easy criticism to refute. Kinetic studies performed by Werkheiser and Markus (1964) suggest that the streptokinase activation of human plasminogen is catalysed by 'Activator 1', a complex formed between streptokinase and 'Proactivator 1', which is a substance postulated to be present in variable amounts in solutions of human plasmin - streptokinase complex discussed above. Thus so far, the proactivator A (Takada) has not been identified as the proactivator 1 entity of Werkheiser and Markus.

2./

2. In addition to the streptokinase activated proactivators discussed above, there is strong evidence to suggest the existence of a plasminogen proactivator which is not activated by streptokinase. To explain this, a short digression is necessary.

The activation of Hageman factor is required for the initiation of three biologically active protein sequences in human plasma. The coagulation sequence proceeds by the activation of Hageman factor upon precursor plasma thromboplastin antecedent (pre-PTA)(Ratnoff et al, 1961) and the kinin-generating pathway by the action of activated Hageman factor upon prekallikrein (Kaplan and Austen, 1970 and 1971). Thirdly, Niewiarowski and Prou-Wartelle (1959) demonstrated that activation of factor XII induced formation of plasmin from plasminogen. Later Iatridis and Ferguson (1962) found that this activation required participation of a second factor. Ogston et al (1969) worked on the isolation and purification of this plasma plasminogen proactivator and named it Hageman factor - co-factor. It has been shown that prekallikrein is also involved in plasminogen activation as the kaolin induced plasmin formation in prekallikrein deficient plasma is reduced (Wuepper, 1972: Weiss et al, 1974 and Saito et al, 1974). The problem then has arisen as to whether or not unactivated Hageman factor-co-factor is identical to prekallikrein. Whereas Kaplan and Austen (1972) and Cochrane et al (1973) deny that kallikrein can transform plasminogen to plasmin both Colman (1969) and Wendel et al (1972) have reported that it can. Studies by Laake and Vennerod (1974) indicate that plasma prekallikrein and factor XII-dependent plasminogen proactivator are identical.

The results, therefore, of current work are confusing and until further evidence is forthcoming, it is probably reasonable to consider Hageman factor-co-factor (plasma plasminogen proactivator) and prekallikrein as separate entities.

To/

To summarise the position to date is difficult, but it is probably fair to say that :-

Firstly, streptokinase forms a complex with human plasminogen and or plasmin which has an activator activity capable of lysing both human and bovine fibrin, and that such a complex is not formed with bovine plasminogen, the reason for which is not known but may presumably be related to some unknown species difference. That streptokinase can, under special circumstances, activate purified human plasminogen without first forming plasmin-streptokinase activator is established.

It has been recently reported that while native streptokinase cannot activate bovine plasminogen, a derivative of streptokinase (SK^1) prepared by interaction of streptokinase with human plasminogen is capable of activating bovine plasminogen (Reddy and Kline, 1975). They present evidence to show that the activator activity of SK^1 towards bovine plasminogen requires the presence of trace amounts of human plasmin. When SK^1 preparations were completely freed of plasmin by passage through a lysine-sepharose column, bovine activator activity disappeared, but human activator activity remained. Addition of human plasminogen to the column-treated SK^1 preparation reinstated the bovine plasminogen activator activity. This work is a further demonstration of the proactivator nature of human plasminogen.

Secondly, evidence exists that a human plasminogen proactivator may be present in human plasma as an $alpha_2$ macroglobulin which, when activated by streptokinase, is capable of converting both human and bovine plasminogen to plasmin.

Thirdly, a proactivator exists which is not streptokinase activatable but which is essential in the pathway for Hageman Factor (or Hageman fragments) to convert plasminogen to plasmin.

TISSUE ACTIVATOR

The earliest suggestion that tissues might produce a substance with fibrinolytic properties came from those working upon tissue culture who observed lysis of the plasma clots used as nutrient medium (Lambert & Hanes, 1911: Fleisher & Loeb, 1915). Fleisher and Loeb claimed that the genito-urinary tract was most active and the liver almost inactive although species differences were found to exist. Demuth and Van Riesen (1928) suggested that the cells released a substance which activated a proteolytic enzyme in the medium, rather than a substance that lysed fibrinogen directly, an opinion substantiated later by Fischer (1946) and Astrup and Permin (1947). In 1947, Astrup and Permin devised the fibrin plate (see Part II). They were able to prove that the fibrinolytic activity present in tissues is indeed due primarily to a plasminogen activator rather than a fibrinolytic enzyme. They further showed that the ability to activate plasminogen was retained by a tissue even after several weeks immersion in a toluene and chloroform solution. The ability then to activate plasminogen is not dependent, or at least not entirely, on the metabolism of the living cell.

Astrup and Sterndorff (1952) showed that saline extracts prepared from human lung and pig brain were active when estimated by the fibrin plate method but no activity was demonstrable on the plasminogen-free heated plate (Lassen, 1952). In addition to the readily saline extractable and labile activator, a much less soluble activator, stable at pH 3 and capable of withstanding heating at 70^{o}C for thirty minutes, is present in greater proportions and can be extracted with 2M potassium thiocyanate (Astrup & Stage, 1952). Albrechtsen (1957) employing this technique demonstrated large amounts of tissue activator in the uterus, adrenal and lymph nodes, prostate, thyroid, lungs and ovary but found the liver almost inactive. No correlation between the activator concentration and age or sex could be established although considerable individual variation was observed.

A/

A histochemical technique (fibrinolytic autography), based upon the fibrin plate method, in which frozen sections of fresh tissue are placed upon thin layers of fibrin made upon glass slides was developed by Todd in 1958. Following incubation and staining, foci of fibrinolysis can be seen in relation to those structures containing tissue plasminogen activator.

EPITHELIAL CELLS

It has been shown that some epithelial cells contain activators of plasminogen. Squamous epithelial cells from human vagina (Tympanidis et al, 1968) and rat vagina (Henrichsen and Astrup, 1967) have been found to be fibrinolytically active. Tympanidis et al (1968) observed that vaginal epithelial cells at a stage of their hormonally regulated evolutionary development acquire the capability to produce and release plasminogen activator. They suggest the prior hormonal influence could possibly explain why some cells are fibrinolytically active while others apparently in the same stage of development are inactive. It would appear that younger cells release activity during the period in which they become mature and begin to undergo degeneration. This was evident from the previous studies in the rat (Henrichsen and Astrup, 1967) where cornified cells were usually inactive while at stages where cells apparently were losing their nuclei, active anuclear cells could be observed among the bulk of inactive cornified cells. This finding is similar to the observation that corneal epithelial cells, normally fibrinolytically inactive, are able to release plasminogen activator after injury or during degeneration (Pandolfi and Astrup, 1967). These findings suggest the possibility of plasminogen activator activity being related to degenerating epithelium. Kwaan et al (1969) consider that this may be the reason for increased fibrinolytic activity in the inflamed rectal mucosa in patients suffering from acute ulcerative colitis. Birn and Fejerskov (1971) appear to have been the first workers to study human oral epithelial cells. Using fibrinolytic autography and controlling/

ling their work by examining endothelial cells under similar conditions, they found that squamous epithelial cells from the buccal mucosa are fibrinolytically active although not to the same extent as endothelial cells. The fibrinolytic activity was mainly associated with the basal, parabasal and spinous cells but occasional foci were related to anuclear cells. In each of their smears, only 10 - 30 pericellular areas of lysis were recorded and the majority of the cells did not show any activity at all. Myhre-Jensen and Astrup (1971) carried out experiments upon squamous epithelium derived from the oral cavity and oesophagus of the rat, guinea pig and rabbit and from this work provided additional evidence of the presence of plasminogen activator in surface epithelial cells of some species and of the 'release' of this activity during normal cellular degeneration or after injury. The activity appeared to originate in the intermediate cell layers of the stratified squamous epithelium, whilst the basal layers and the most superficial cornified layers were inactive. Fibrinolysis also seemed most marked in areas of cellular detachment. Wunschmann-Henderson and Astrup (1972) using a combination of Papanicolaou's staining technique and fibrinolytic autography demonstrated the presence of fibrinolytic activity in buccal smears to be correlated with specific states of cellular maturation. The highest percentage of fibrinolytic cells was found among the groups of younger desquamated epithelial cells.

THE SEQUENTIAL ACTION OF PLASMIN UPON FIBRIN AND FIBRINOGEN.

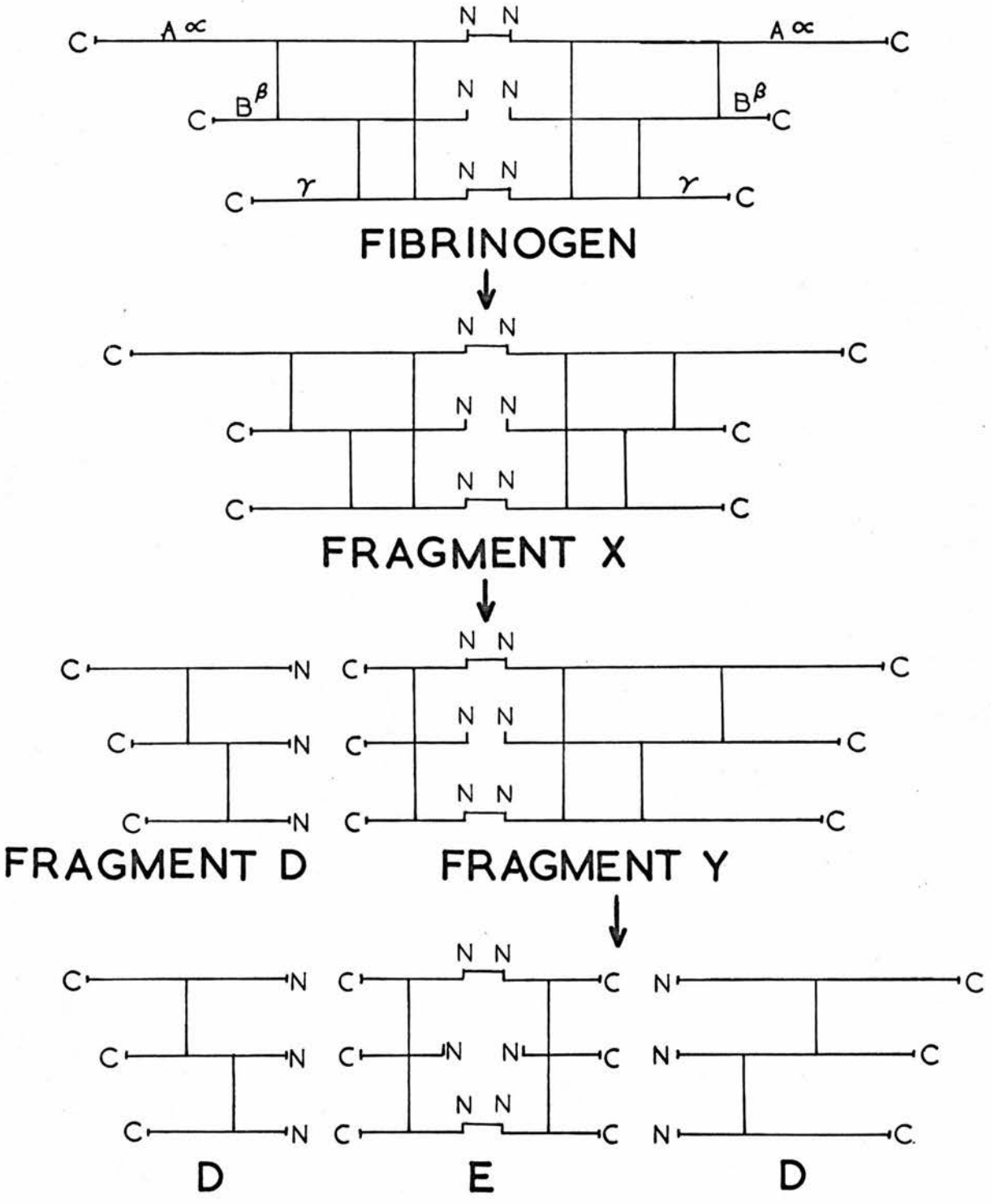
Human fibrinogen is composed of two pseudo-identical monomers joined by a number of symmetrical disulphide bonds (Soderqvist and Blombäck, 1971). Each monomer consists of three polypeptide chains the respective molecular weights of which are 63,800, 56,000 and 47,000. These molecular weights are consistent with the estimated molecular weight of 340,000 of the dimer (McKee et al, 1966). The N-terminal segment of the fibrinogen molecule contains eleven disulphide bridges which form a firm "Disulphide Knot", the primary structure of which consists of about 16% of the whole fibrinogen and has been completely elucidated.

Very little is known as yet about the remaining C-terminal part.

Plasmic hydrolysis of fibrinogen produces high molecular weight Fragments. Nussenzweig and Seligman (1960) identified and partly characterized five terminal digestion products called Fragments A, B, C, D and E. The physiologically early degradation products which are potent anticoagulants have been isolated from partial plasmin digests and designated as Fragments X and Y. A scheme for the asymmetrical fragmentation of fibrinogen by plasmin has been proposed by Marder et al, 1972 and is reproduced in Diag. 2.

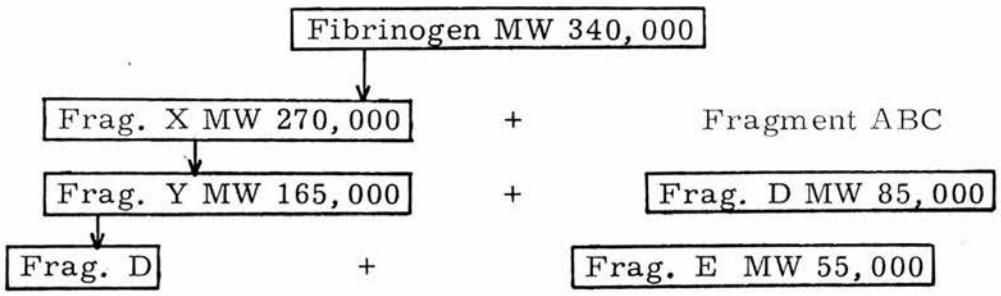
Diag. 2

STRUCTURE OF F.D.P.



(MARDER et al, 1972).

DEGRADATION PATHWAY OF FIBRINOGEN
BY PLASMIN



Marder, 1972.

In the early phase of plasmic hydrolysis, the beta and gamma chains of fibrinogen lose small molecular weight peptides at the N- terminal and C- terminal ends, respectively. Large polypeptides are progressively being split from the C-terminal part of the alpha chain. Fragment X was found to be a heterogeneous population of molecules differing in the length of their alpha chains. On further digestion, one pair of disulphide-bound beta and gamma chains is released from the fragment X, giving rise to fragment Y, which in turn splits into another beta-gamma dimer and fragment E. The beta-gamma dimer corresponds to Fragment D and Fragment E may be identified at least partially with the disulphide knot (Furlan and Beck, 1972). Fragments A, B, C, D and E are resistant to further digestion by plasmin. Each fragment represents an intact portion of fibrinogen and it is not surprising, therefore, that they all have similar antigenic activity - the two major determinants being the D and E antigens. Fibrinogen, Fragment X and Fragment Y each carry both determinants while Fragments D and E carry only the one respectively. Thus an antiserum raised to fibrinogen or a mixture of the products (i.e. with both anti-D and anti-E activity) will react with all fragments. This common immunological activity is the basis of many of the assay systems for FDP. The degradation of fibrin by plasmin is believed to follow the same pathway as above and so far using routine immunological methods, no significant/

nificant difference has been demonstrated between fibrin products and fibrinogen products. However, a very recent radioimmunassay technique does detect slight differences between these two groups (Plow and Edgington , 1972).

Fibrin/fibrinogen degradation products have been demonstrated in the serum of 95% of normal healthy individuals to a level of five microgrammes/ml., these circulating fragments being identical to those prepared and described using in vitro techniques (Das et al, 1967). An increase in this level is caused by one of two ways, either by primary activation of the fibrinolytic system giving rise to FDP or by secondary activation stimulated by increased coagulation and resulting in the breakdown of fibrin to FDP.

THE FIBRINOLYTIC MECHANISM IN VIVO

The basic components of the fibrinolytic system, plasminogen, plasmin, activators and proactivators of plasminogen, but not plasminogen inhibitors have been discussed. It is, therefore, appropriate now to consider in a simplified manner how these parts may be integrated to form the whole. Two main mechanisms of fibrinolysis in vivo have been proposed: the Sherry hypothesis, and the plasmin-inhibitor complex hypothesis advocated by Ambrus.

The Sherry Hypothesis

The hypothesis (Sherry et al, 1959) is widely accepted as a working hypothesis to explain how the relatively non-specific proteolytic enzyme, plasmin, is largely restricted in vivo to a single highly specific action, digestion of fibrin.

In a system that includes both plasma and clot, plasminogen exists in two phases. Plasminogen in the plasma represents the soluble phase and plasminogen in the clot the gel phase. Plasminogen activation in the soluble phase, produces no effects on susceptible substrates in the plasma because plasma antiplasmin rapidly inhibits plasmin as it is formed. However, rapid plasma plasminogen activation may, by a temporary overwhelming of the antiplasmin mechanism, permit the appearance of free plasmin in the circulation with digestion of a variety of substrates including fibrinogen and other coagulation factors and hence produce a haemorrhagic state.

Plasminogen activation in the clot or gel phase, where the effect of inhibitors on adsorbed activator and plasminogen is weaker than in the plasma, produces a different result. Under these conditions because of the spatial relationship of plasminogen and fibrin, plasminogen activation produces fibrinolysis. Lytic activity then is considered as a function of clot plasminogen content. Sherry suggests that the main function of plasma plasminogen is to endow any intravascular fibrin that may form with the means to bring about its own lysis when activator/

activator is either adsorbed upon it during its formation or subsequently diffuses into it from the plasma. Plasminogen is known to have a strong affinity for fibrinogen and fibrin (e.g. Blombäck and Blombäck, 1956) and activator is also adsorbed onto fibrin (Fearnley et al, 1952).

The Plasminogen-Inhibitor Complex Mechanism (Ambrus)

Ambrus and Markus (1960) have suggested that plasmin may circulate in the plasma as a dissociable complex with antiplasmin, and that fibrin competes for the plasmin with antiplasmin: in this way, there is dissociation of the plasmin-antiplasmin complex and specific localization of plasmin on fibrin. Thus effective substrate specificity is conferred on plasmin.

Objections

The Sherry hypothesis is almost universally accepted but evidence is accumulating to suggest plasminogen is not, in fact, adsorbed to fibrin when it forms and that the only plasminogen present in a thrombus is that contained in the trapped plasma (Hedner et al, 1966: Ogston et al, 1966). De Witt (1964) and Fantl (1962) demonstrated that the plasminogen contect of plasma and serum is identical. A further objection is raised by the observations of McNicol et al (1965) and by Dalal et al (1969) that in artificial thrombi perfused with plasminogen activator in saline, full thrombolysis may not occur unless the perfusion medium is plasminogen-enriched.

Sharp et al (1972) had demonstrated that antiplasmin is also present in trapped plasma within a thrombus.

The objections outlined above do themselves raise problems. One of these is succinctly put by Sharp, "If it be accepted that plasminogen is not bound to fibrin and that in the fibrin mass, plasminogen and antiplasmin are contained only in the trapped plasma, how can activator induce thrombolysis?"

A/

A variation of the Ambrus hypothesis has been forwarded by Sharp et al (1972). It is that activator can link with an $alpha_2$ globulin in the plasma or in the trapped plasma of a thrombus and generate from the plasminogen, a plasmin that is protected from the action of antiplasmin, the receptor linkage for antiplasmin apparently being blocked by the $alpha_2$ globulin.

Confirmatory evidence for any of these hypotheses is lacking but there are sufficient problems inherent in each to make the research worker use them only as working models always ready to be modified as new facts come to light.

THE ROLE OF THE FIBRINOLYTIC SYSTEM IN VIVO

There is evidence to support the view that urokinase has a physiological role in maintaining the patency of the urinary tract (McNicol et al, 1961) and a similar function may exist elsewhere as plasminogen activator is found in milk (Astrup & Sterndorff, 1953), tears (Storm, 1955), saliva (Albrechtsen & Thaysen, 1955), and seminal fluid (Albrechtsen, 1958). Tissue fibrinolytic activity is localized in sites at the angle of the anterior chamber of the eye and is related to the canal of Schlemm (Kwaan & Astrup, 1963). In the foetus and newborn infant, outflow of aqueous humour has not yet been established and the fibrinolytic activity at this stage is also absent (Pandolfi & Kwaan, 1967). The diffuse superficial fibrinolytic activity of normal endometrium increases during the menstrual cycle and is maximal at the time of menstruation in the shed endometrium (Todd, 1964). In women with menorrhagia, Rybo (1966) reported that the levels of plasminogen activator in the endometrium were higher than those in women with normal menstrual loss.

Plasma fibrinolytic activity may be reduced in patients suffering from diseases associated with thrombosis (McNicol & Douglas, 1972). Nestel (1959) found reduced plasma fibrinolytic activity in a group of patients with intermittent claudication as compared with healthy controls. Ellison and Brown (1965) observed that in patients with a previous episode of pulmonary embolism systemic fibrinolytic activity was low. In patients suffering from deep venous thrombosis, Pandolfi et al (1969) found reduced systemic fibrinolytic activity, and plasminogen activator in their vein walls was also reduced. High levels of naturally occurring fibrinolytic inhibitor may be associated with widespread thrombotic disease (Nilsson et al, 1966: Brakman et al, 1966).

In 1970, 63,640 deaths were recorded in Scotland and of these 33,958 (53.4%)/

(53.4%) were attributed to the circulatory system. Heart disease accounted for 21,497 and cerebrovascular disease for 9,952 deaths. To put these figures in perspective in terms of death rate per 100,000 of the population the neoplasia figures are 271 male and 217 female compared with 460 male and 370 female for heart disease (Annual Report of the Registrar General for Scotland 1970: Part 1. 'Mortality Statistics' No. 116).

The formation of fibrin is a part of normal tissue repair but in order to restore normal conditions, the fibrin has to be dissolved. In large infected wounds where great numbers of leukocytes are present, fibrin may be removed chiefly by a cellular mechanism (Astrup, 1968) but as the weak lytic effect of leukocytes is caused predominantly by a protease (Astrup et al, 1967: Wunschmann-Henderson et al, 1972) they probably play a secondary role, the principal role being played by the capillary endothelial cells at the commencement of and during organisation as these cells produce considerable quantities of plasminogen activator (Astrup et al, 1967). The fibrinolytic activity of human joint tissues, synovial membranes and fibrous capsular tissues is low (Astrup and Sjolin, 1958: Nikitin et al, 1968) as is the content of tissue thromboplastin. These findings may explain why haemophilic patients bleed easily into their joints and why extensive fibrosis is a common finding in these joints.

Cliffton and Grossi (1955) were able to demonstrate that with carcinomas of the breast which were activators of human plasminogen in vitro, there was a higher incidence of positive axillary nodes in vivo although anti-coagulants and fibrinolysins have been shown to reduce the growth and spread of malignant tumours in experimental animals (Wood et al, 1961: Cliffton and Agostino, 1965). Ogston et al (1971) observed that the presence of hepatic metastases was associated with the reduced plasminogen levels in association with low activator levels. The significance of this is not yet known.

Thus/

Thus the process of fibrinolysis and fibrinolytic activity is ubiquitous not only anatomically, but also by its participation in the classical divisions of pathology, inflammation and neoplasia. If to this is added a physiological role, then the importance of the system is self-evident.

CHAPTER II

(i) SALIVA

The salivary glands of man consist of three paired, large glands (parotid, submandibular and sublingual) and the smaller glands (labial, lingual, palatal and buccal). In this work, the secretion of any one of these glands is prefixed by the name of the gland, e.g. 'parotid saliva'.

Naturally pooled salivary secretions complete with cellular matter, gingival fluid etc. are referred to as 'mixed native saliva' or MNS. Cannulation or cupping enables the collection of a specific secretion and if two such secretions are pooled, the resulting fluid is called 'artificially mixed saliva' or AMS.

The salivary secretions flow into a cavity which is open to the atmosphere and is therefore heavily contaminated with bacteria. The cavity is lined by oral mucous membrane which continually sheds epithelial cells and in the dentate a variable amount of gingival fluid containing leukocytes is added to the MNS. From time to time during the day, a certain amount of food debris will also be in suspension. Thus the fluid that bathes the teeth and oral mucosa (MNS) is of a very variable composition, the major constituents being :

1. Salivary secretions.
2. Epithelial cells.
3. Bacteria.
4. Leukocytes and gingival fluid.
5. Food debris.

With the exception of food debris, these were all considered in relation to the fibrinolytic activity of MNS.

Problems/

Problems of investigation

MNS is the fluid that actually bathes the teeth and mucosa and ultimately it must be the action of this fluid that has to be assessed when consideration is given to the effect saliva has on, for example, blood clotting in a tooth socket or digestive action within a food bolus.

Centrifuged MNS gives lower figures for some constituents than does uncentrifuged (Jenkins, 1966) but on the other hand, if the suspended matter is not removed analytical procedures are much more difficult. For practical purposes, this separation must take place and two entities recognised, the supernatant and the pellet. The separation itself is not without hazard for ultracentrifugation can break up cells but such centrifugation is required as many of the suspended particles in saliva are very small indeed.

Qualitative examination of supernatant saliva is generally straight forward, and the absence or presence of an enzyme, electrolyte or vitamin determined, but accurate quantitative analysis is extremely difficult. The contributory secretions differ in composition and relative contribution. The individual contributary secretions do themselves vary in composition with conditions such as rate of flow, type and intensity of stimulus, time of day or week, and the nature and time of the last meal prior to collection. The presence of living bacteria, if they have not all been removed by centrifugation, and the spontaneous loss of carbon dioxide after collection causes changes in the composition on standing and thus the accuracy of some analytical techniques depends upon the length of time elapsing between collection and analysis.

Collection of 'pure' saliva by cannulation and cupping is full of pitfalls. In this work, the writer has had recourse to collect parotid and submandibular/sublingual saliva and has found many problems not always mentioned by other workers. The impression given by some workers/

workers is that cannulation or cupping will provide a 'pure' secretion. Examination of the secretions collected in this work frequently revealed the presence of desquamated epithelial cells and cell fragments which necessitated removal by centrifugation. When a Curby cup was in situ and apparently well attached, it was interesting to observe that when a few drops of Cochineal food dye was placed by pipette into the subject's mouth, there were occasions when it would not have been necessary to resort to a colorimeter to determine the presence of the dye in the 'pure' parotid saliva.

The writer cannot claim ever to have collected unstimulated saliva. With practiced people in the Laboratory who were used regularly for pilot experiments, it became easier to collect saliva and a practice effect, although never measured in this work, was certainly apparent. At best, a distinction could be made between deliberately stimulated saliva and very carefully collected relatively unstimulated saliva. To extrapolate one's findings from a study population to the community is not without danger. The writer found that although he took most of his subjects at random from the student body, the criteria of "no clinical evidence of gingivitis" removed several from the samples and when collection did begin approximately one third of the subjects proved quite hopeless for obtaining an adequate sample of every saliva. Parotid collection proved most difficult, mainly with the unstimulated flow, and even MNS collection, naively assumed at the start of the work to be straightforward frequently provided more froth than fluid.

Variables such as age, sex, time of collection and time of the last meal are data that can be recorded objectively, but many of the above variables cannot be assessed.

From the comments made in the literature and from what the writer experienced during preliminary investigations, the following salivas were chosen for this work.

1./

1. MNS. This is the fluid that bathes the teeth and mucosa and is ultimately the substance acting upon a blood clot in the mouth. Every effort was made to collect it under two conditions; relatively unstimulated being as near as practically possible to representing the fluid bathing the oral structures at rest, and stimulated when large quantities of MNS or its supernatant were required for qualitative analysis.

2. The supernatant of MNS. Generated from stimulated MNS first by low speed centrifugation at 4154 g. and then a further centrifugation of the supernatant at 36, 664 g. Qualitative analysis was carried out upon the supernatant for soluble activator(s) and plasminogen.

3. Isolated secretions from parotid and submandibular/sublingual salivary glands in order to obtain information concerning the source of soluble activator or of plasminogen. Less than 8% of the salivary secretions arise from the minor salivary glands (Dawes and Wood, 1973) and therefore it is reasonable always to examine the paired glands first.

4. Salivary pellet. In the analytical sieve this may be taken as consisting of four main parts, viz. mucus, bacteria, epithelial cells and leukocytes. These constituents were studied separately, and following isolation were also recombined with the supernatant of MNS to indicate a possible role in vivo.

Details of the collection of saliva are contained in the Methodology.

(ii) THE FIBRINOLYTIC ACTIVITY OF SALIVA

Using the bovine fibrin plate much improved by Astrup and Mullertz (1952), Albrechtsen and Thaysen (1955) first described the fibrinolytic activity of saliva. They found that plating both parotid and mixed native saliva (MNS) produced only "slight" activity, but that the addition of streptokinase to the salivas enhanced activity considerably. Heated fibrin plates were considered as synonymous with plasminogen-free plates and upon these they found no activity either with or without the addition of streptokinase, and from this deduced the absence of both plasminogen and a fibrinolytic enzyme. Human fibrinogen used in the assessment of salivary fibrinolytic activity appears to have been first used by Taylor et al (1964) when saliva was substituted for euglobulin in a clot lysis time experiment. They found lysis of the clot occurred rapidly in all samples tested being most rapid in MNS and parotid. MNS supernatant while producing lysis, did so less rapidly. No details of saliva collection, centrifugation or control other than with saline are given. They concluded that a fibrinolytic enzyme must be present together with a streptokinase-activated proactivator, but not a plasminogen activator. They also found that when the pellet was tested for lysis, in most instances lysis did not go to completion in twenty-four hours. Dolci (1965) using thrombelastographic techniques deduced the presence of both activator and pro-activator, and Tortelli (1967) returning to bovine fibrin plates concluded that MNS has a fibrinolytic activity that is enhanced by streptokinase but that MNS supernatant, parotid and submandibular saliva do not cause fibrinolysis except with the addition of streptokinase. He concludes that the fibrinolytic activity must be related to the pellet (cf. Taylor et al, 1964). Schulte published eleven papers between 1964 and 1967 concerning saliva and fibrinolysis and in 1970 presented a summary of his findings. His experimental methods were confined to the bovine fibrin plate and thrombelastographic techniques. He claims the presence of an activator in small quantities and pro-activator/

vator in much larger amounts. Pro-activator was always demonstrable but activator was not present on some days. Plasminogen and plasmin are absent. Schulte and Gewalt (1964) found that sex had no influence upon activator or pro-activator activity, but Schulte and Goens (1967) found that pro-activator and activator activity increase during menstruation.

Nitta et al (1967) produced the first physicochemical study of the properties of the fibrinolytic substances in human saliva. They undertook this study because they found (as the writer has discovered also) that the results of previous workers were "not always coincident except concerning the presence of pro-activator." Their basic methodology is confined, however, to heated and unheated bovine fibrin plates, the testing of salivas upon these plates with and without streptokinase, and the examination of saliva following fractioning on a Sephadex G75 column. From their work, they conclude the absence of an activator and the presence of large amounts of pro-activator and lesser amounts of plasminogen. They found the fibrinolytic substances are inhibited by epsilon-amino-caproic acid (eACA) and in very low concentration (whole saliva fibrinolysis inhibited with 2^{-6} MeACA). Schulte (1970) however claims, "The fibrinolytic actions of human saliva can be inhibited by eACA, but very high local concentrations are required". The pro-activator and plasminogen Nitta et al (1967) believe are activated in vivo by bacterial contamination, but present no evidence for this, while Tortelli (1967) claims the bacteria in the pellet are completely inactive. The results of Sephadex fractionation on G75 (Nitta et al, 1967) are interesting since they show the presence of a streptokinase-activated pro-activator emerging with a molecular weight in excess of 50 - 70,000.

More recent work of Wedgwood (1970) has failed to clarify the very muddled picture that the literature provides. Adapting the euglobulin lysis time method of Taylor et al (1964) he found the end point very hard to read because of the turbid nature of saliva and he concludes/

cludes that the activator must be "a part of, or adsorbed to the mucoprotein moiety of saliva".

It appears from the literature that only the most general conclusions can be drawn.

1. MNS may contain a weak plasminogen activator(s). The results on bovine fibrin plates are disputed and human fibrin plates do not appear to have been used up to date (1973).

2. There is complete agreement that a streptokinase-activated proactivator is present in MNS.

3. The source of both activator and proactivator in MNS is unknown and conflicting views exist concerning the importance or otherwise of the salivary pellet.

4. One attempt has been made to provide some physiocochemical information about the proactivator. It has been given a molecular weight in excess of 70,000 and is streptokinase activated which is strongly suggestive of being plasminogen but it has never been demonstrated directly as such.

A neat summary of the position in 1967 is contained in the paper by Nitta et al (1967) and this is reproduced below. At the commencement of this work, the position concerning knowledge about the fibrinolytic activity of human mixed native saliva is adequately described in this table. No significant information has been published since.

Fibrinolytic Substances in Human Saliva (Nitta et al, 1967)

Fibrinolytic/

Fibrinolytic Substances

Investigator	Date	Plasmin.	Plasminogen Activator	Pro-activator	Plasminogen	Antiplasmin
Albrechtsen et al	1955	-	+	+	-	-
Taylor et al	1964	-	-	+	-	
Schulte	1964	-	+	+	-	
Nagai et al	1966	+	+	+		
Nitta et al	1967	-	-	+	+	-

Key (+) Present (-) Absent

(iii) OBJECTIVES

To provide answers to the following three questions :-

1. Does human mixed native saliva possess specific plasminogen activator activity?

2. If activator activity is present, what is its source?

3. What is the nature of 'proactivator' in saliva?

PART II

PRELIMINARY EXPERIMENTS

Chapter 1 The Fibrin Plate.

Chapter 2 Experiments :

- Experiment 1. Optimum incubation time for solutions applied to fibrin plates.
- Experiment 2. Sensitivity of standard human and standard bovine fibrin plates.
- Experiment 3. Interplate variation in sensitivity and its correction.
- Experiment 4. MNS. Plasminogen activator activity or non-specific proteolysis ?
- Experiment 5. Normal range of plasminogen activator activity in MNS.
- Experiment 6. Stability of plasminogen activator in MNS.
- Experiment 7. Intra-personal variation in plasminogen activator activity in MNS.

Chapter 3 Summary of Conclusions to Preliminary Experiments.

CHAPTER I

THE FIBRIN PLATE

The fibrin plate was originally described by Permin (1947) and later improved by Astrup and Mullertz (1952) who suggested optimal conditions for its use in the estimation of Plasmin. It has proved useful for the estimation of the plasminogen activator in urine, urokinase (Smyrniotis, Fletcher, Alkjaersig and Sherry, 1959: Holemans, McConnell and Johnston, 1966) and also for the activity of various proteolytic enzymes (Astrup and Alkjaersig, 1952: Haverkate and Traaf, 1974).

A solution of fibrinogen is clotted with thrombin in a Petri dish and on the fibrin so prepared is placed a small amount of the substance to be assayed. The plate is then incubated for a set period and lysis is manifest as a clear circular zone, the area of lysis (being conveniently defined as the product of two perpendicular diameters) giving a measure of the fibrinolytic activity of the substance tested.

Three variants of the fibrin plate have been developed: namely, plates prepared with bovine fibrinogen, with human fibrinogen, and bovine or human fibrin plates heated to destroy the plasminogen content in order that plasmin activity might be directly assayed (Lassen, 1952). These variants have all been used to investigate the fibrinolytic activity of human saliva (Albrechtsen and Thaysen, 1955: Taylor et al, 1963: Schulte and Gewalt, 1964: Dolci, 1965: Nitta et al, 1967: Tortelli, 1967: Wedgwood, 1970).

The fibrin plate has some disadvantages. Salt and hydrogen ion concentration have to lie within the limits for which thrombin can form an adequately polymerised clot with fibrinogen. This fact imposes a pH range of 6.8 - 8.2 and an ionic strength not higher than that found in an 0.45 Molar solution of sodium chloride (Wolf, 1972). /

1972). The test system is therefore rigid. When fibrin plates are used for the estimation of plasminogen (measured by determining optimum generation of plasmin from the areas of lysis after plasminogen kinase interaction) the plasminogen originally present on the fibrin plate must first be inactivated. This is normally achieved by heating the plates (Lassen, 1952) but this decreases the sensitivity of the fibrin substrate towards subsequent digestion by plasmin (Alkjaersig, Fletcher, and Sherry, 1959: Wolf, 1968: Shiba, Igarashi and Takeuchi, 1973). In the light of this information, the heated plate was not used in this investigation and a direct method for plasminogen estimation was developed.

Unheated fibrin plates often show spontaneous lysis during storage prior to use. The addition of calcium chloride at an end concentration of 0.001M. enables fibrin plates to be stored for up to seven days at 4^{o}C. with no apparent alteration in sensitivity (Tsapogas and Flute, 1964). For standardisation, all plates used in this work were freshly prepared. It was found that in the attempt to be economical, it was not possible to reuse disposable Petri dishes without first treating them with a silicone solution, otherwise a high incidence of spontaneous lysis occurred. Conversely, by using this silicone treatment in conjunction with calcium chloride spontaneous lysis was eliminated. (For details of Silicone treatment, see Methodology, p XXIII).

Recent work (Noren, Ramstrom, & Wallen, 1975) has demonstrated the elegance and economy of using fibrin agar plates for measuring saliva fibrinolytic activity. This work is a development of the original system described by Heimburger and Schwick (1962) and improved by Wolf (1968), and Stuckey & Wolf (1969). However, it was considered wise not to change to the agar system towards the end of the investigation and thus the standard fibrin plate was used throughout, modified only by the addition of calcium chloride. Very recently/

recently, Haverkate & Brakman (1975) have expressed concern about adding agar to fibrin plates as they fear it might influence the results. They do not say why they think this. (For details of the preparation of fibrin plates, see Methodology, p I).

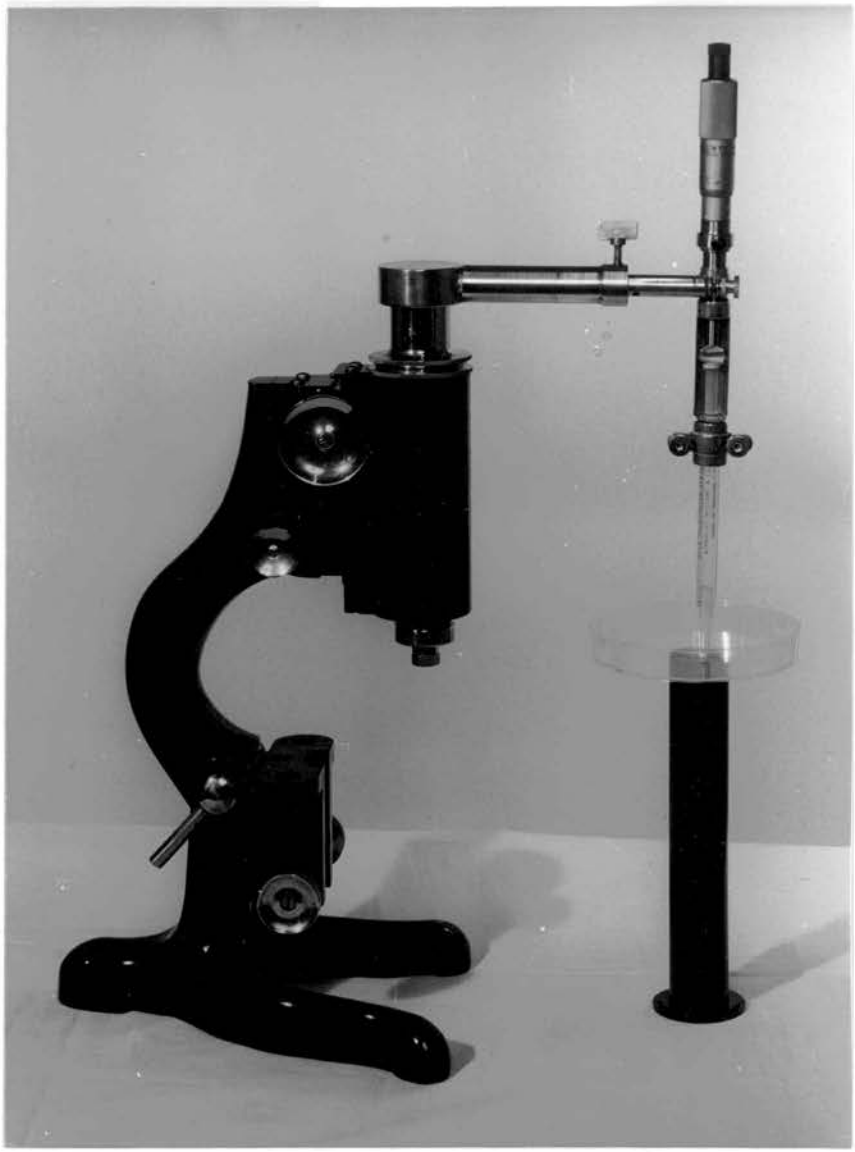
The application of solutions to fibrin plates

The application of non-viscous liquids such as urokinase is readily accomplished using a glass blow out pipette and a neat circular drop may be obtained on the plate. Mixed native saliva and submandibular saliva are so viscous that the drop size is very irregular and because the drop hangs on the pipette, it has to be tapped off achieving, at best, an irregular and inaccurately placed blob. The method is totally unsatisfactory.

In order to achieve accurate reproducible drops, a micrometer syringe was attached to a modified microscope base (Figure 1) It was possible to deliver a drop of the desired volume, but not as neatly as might be expected, as the drop tended to hang from the needle point and required a tap to be released. The alternative method was to lower the drop on to the plate and then lift the needle, but this generally resulted in either damaging the plate or lifting some of the viscous liquid back up with the needle. The method was not as satisfactory as had been anticipated and it was also very time consuming considering that frequently upwards of forty plates were set up at any one time. To be used regularly, a large supply of disposable needles and glass syringes would have been necessary. The method was abandoned.

Joyce & Tyler (1973) carried out a systematic examination of various dispensing pipettes and concluded that the most accurate in the range required for fibrin plate experiments, 0 - 200 micro litres, was the Gilson Pipetman P200. The disposable tips are quickly changed, the adjustments necessary to alter the volume delivered/

FIGURE I



MICROMETER SYRINGE ATTACHED

TO MODIFIED MICROSCOPE

BASE

delivered are simple, and of considerable importance is the "second pressure" which allows viscous fluids to be completely expelled from the pipette. Initially, the drops delivered with this pipette were irregular, but with practice neat reproducible circles were achieved. In this work, all drops were delivered using the Gilson Pipetman P200.

Interpretation of lysis

After incubation, a blue dye (bromothymol blue) is added to the lysed area and the now clearly demarcated area of lysis is measured by calculating the product of two perpendicular diameters measured by placing the transparent plate over centimetre graph paper. It is possible to measure to half of one millimetre by this method. The bromothymol blue is made up in ethyl alcohol (for details, see Methodology, p II) and if it is applied as a neat solution to a human fibrin plate directly, and not into an area of lysis, it will itself lyse the fibrin where it is applied within minutes. It is very important, therefore, to apply the dye to one area of lysis at a time and read it immediately. This is simple as the dye disperses rapidly within the lysed fibrin. If the operator now applies a drop of the dye to the other two or three lysed areas on the plate and remeasures the first lysed area, it will be found that the initially clearly demarcated perimeter is hazy and its external diameter is anywhere between a half and one millimetre increased in size. It was observed that this increase applied regardless of the size of the initial lysis and thus the larger the initial size of lysis, the more important it became to read the plate at once. The haziness of the border is due to the diffusion of the dye into the fibrin, and if the plate is left overnight, the whole plate will be pale blue by morning.

It is the experience of the writer that determining what actually is lysis is not always easy. If a solution of 5 Ploug units/ml. of urokinase/

kinase is doubly diluted and applied as 0.03 ml. drops to fibrin plates, then interpretation of lysis becomes difficult below a strength of 6.25% (Part II, Expt. 2). When a smaller concentration is applied to human plates, an area of lysis is usually obtained but it is shallow and only sometimes penetrates the three millimetre thickness of the fibrin on which occasion the penetration is that of an inverted cone. (Upon bovine plates, the area is more clearly demarcated and the walls of the lysed area are nearly parallel). At still smaller concentrations, there is a disturbance of the surface indicated by the dispersion of the dye, but no actual penetration of the fibrin through to the base of the Petri dish. This activity is not recordable, but it is different to the behaviour of the negative control solutions. When dye is applied to an area upon which tris buffer has been incubated, the dye momentarily remains as a blob before spreading very slowly and with no characteristic whirling action. Very weak solutions of known activators upon bovine plates occasionally produced an area of opacity on the plate and rarely odd phenomena occurred such as concentric areas of lysed and unlysed fibrin and a "double well" effect in which clearly definite lysis had taken place over quite a wide area with a much smaller central area of lysis which penetrated through to the plate.

In view of these variables, it was necessary to establish criteria by which lysis could be interpretated.

1. Lysis — Lysis is present where there is penetration through the fibrin to the base of the Petri dish and when in conjunction with this, the applied dye demarcates a clear area that can be easily measured. Experience has shown that on human fibrin plates, the minimal size of lysis that can be measured with confidence is 64 sq. mm. (8 x 8 mm.) Bovine fibrin plates allow readings down to 49 sq. mm. (7 x 7 mm.)

2./

2. Probable Lysis — This term indicates that there has been a disturbance of the fibrin immediately below where the test solution has been applied and that a liquid is present in which dye has dispersed, but there is no penetration through to the base of the plate, neither can the area be accurately determined. This applies to both human and bovine fibrin plates and is indicated in result tables by an *.

3. No Lysis — No disturbance of the surface of the plate whatsoever.

CHAPTER II

Experiment 1

Object: To establish the optimum incubation time for test solutions applied to fibrin plates.

Materials:

1. Standard human fibrin plates, (SHFP). Methodology, p. I
2. Urokinase. 5 Ploug units/ml. in tris buffer pH 7.8, 0.15M.
3. Negative control. Tris buffer pH 7.8, 0.15M.

Method:

Three drops of 0.03 ml. of urokinase and an 0.03 ml. drop of tris buffer were plated on each of seven SHFP. The plates were incubated at 37°C. After eighteen hours, plate number 1 was removed and the areas of lysis recorded. The remaining plates were removed at hourly intervals and the areas of lysis recorded. The experiment was repeated twenty times.

Results:

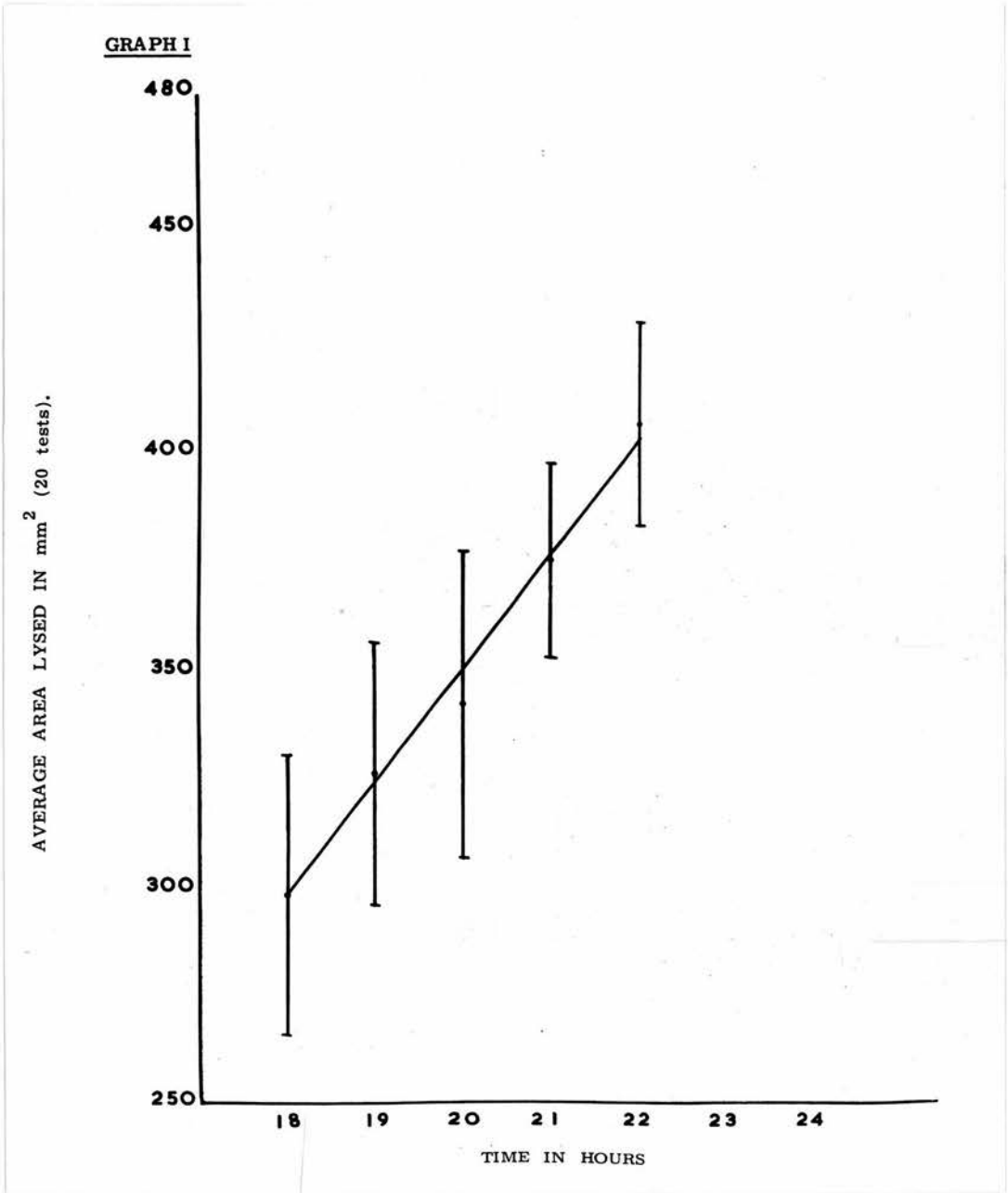
These are recorded in Table 1 (Appendix 1) and on Graph 1. The negative control solution did not produce lysis.

Summary of Results:

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. of urokinase (5 Ploug units/ml.) upon SHFP after incubation at 37°C from 18 to 24 hours.

	Incubation Time (hours)						
	18	19	20	21	22	23	24
Mean (20 tests)	299	327	343	376	407		
S.D.	32	30	35	22	23		

Mean/



Progress curve for urokinase activated human plasminogen (contained within a standard human fibrin plate) from 18 to 24 hours. Urokinase: 0.03 ml. drop volume. Conc. 5 Ploug units/ml. pH 7.8 Temperature + 37°C.

Mean values for 23 and 24 hours incubation are not given as several plates showed total lysis.

Discussion:

Graph 1 shows the progress curve for urokinase activated human plasminogen (contained within a standard human fibrin plate) between 18 and 24 hours incubation. (The line of best fit has been placed in accordance with the method of least squares (Croxton & Cowden, 1963)). The curve clearly indicates increased lysis (plasminogen activation) with increased incubation time.

With the appearance of totally lysed plates at 24 hours incubation, a shorter incubation time is indicated.

A small range of readings was obtained at 19 hours with a standard deviation of only thirty and this was also a convenient time for experiment planning. This experiment was conducted using only standard human fibrin plates because these alone would be used to assess activator activity. A similar time of incubation would be used with bovine plates in the assessment of proactivator activity in order to standardise incubation times.

Conclusion:

Standard human and bovine fibrin plates would be incubated for 19 hours at $37^{o}C$.

Experiment 2

Object: To assess the sensitivity of standard human and bovine fibrin plates at 19 hours incubation.

Materials:

1. Standard human and bovine fibrin plates. Methodology, p. I & IV
2. Urokinase. 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
3. Dilutant and negative control solution. Tris buffer, pH 7.8, 0.15M.

Method:

Urokinase was serially diluted in tris buffer to provide solutions with a range of concentration from 5 Ploug units/ml. (100%) - 0.08 Ploug units/ml. (1.56%). For each test, seven bovine and seven human plates were prepared. Upon plate 1 of each type were placed three drops (each 0.03 ml.) of urokinase at a concentration of 100%. In the centre of each plate was placed one similar drop of tris buffer as a negative control. This procedure was repeated upon plates 2 - 7 but using a different dilution of urokinase on each plate. The plates were incubated for 19 hours. The average of the three lysed areas per plate was recorded as the area lysed in square millimetres. The experiment was repeated twenty times.

Results:

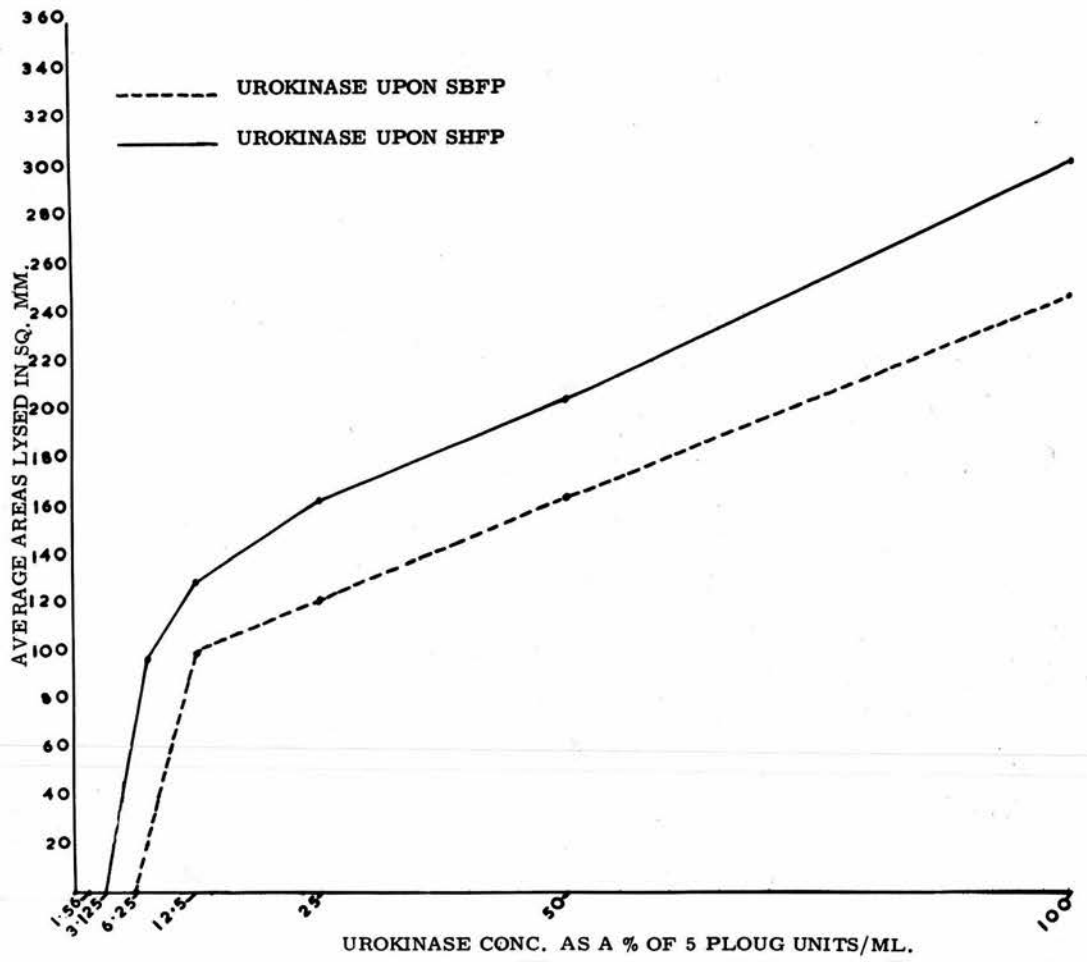
These are recorded in tables 2 and 3 (Appendix 1) and on graphs 2 and 3.

The negative control solution did not produce lysis.

Summary of Results:

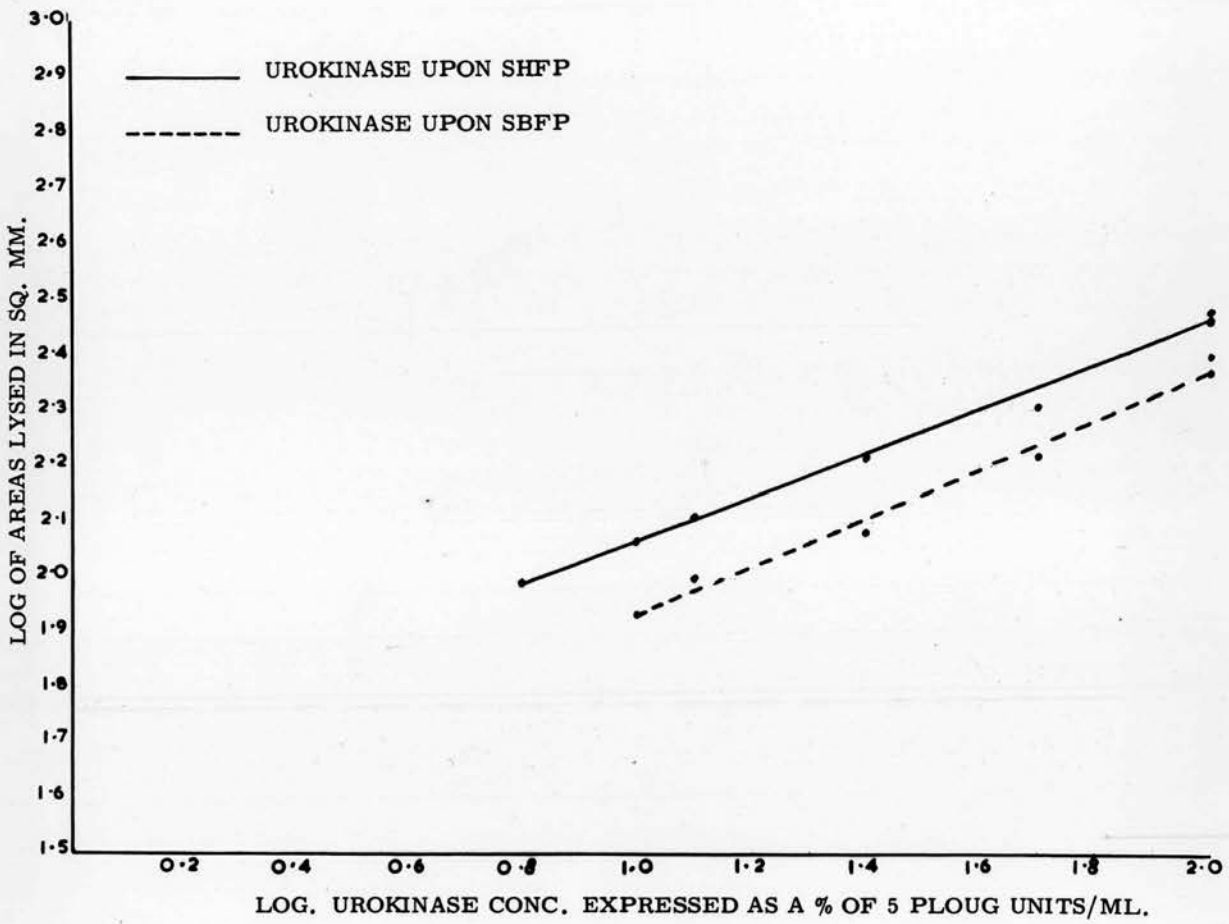
Average/

GRAPH 2.



Average areas lysed by urokinase upon SHFP and SBFP after 19 hours incubation at 37°C.

GRAPH 3.



Log of the average areas lysed by urokinase upon SHFP and SBFP after 19 hours incubation at 37^{o}C.

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of urokinase doubly diluted to a concentration of 1.56% upon (a) SBFP and (b) SHFP after 19 hours incubation at 37°C.

	Urokinase concentrations as a percentage of 5 Ploug units/ml.						
	100	50	25	12.5	6.25	3.125	1.56
(a) SBFP							
Mean (20 tests)	246	164	122	100	0	0	0
S.D.	19	20	11	7	0	0	0
(b) SHFP							
Mean (20 tests)	300	204	163	130	98	*	0
S.D.	56	38	36	28	9	0	0

* Probable lysis.

Discussion:

From these results, it is evident that measurable lysis occurred with urokinase at dilutions greater than 12.5% of the standard urokinase solution upon SBFP, and at dilutions greater than 6.25% upon SHFP after 19 hours incubation at 37°C.

Probable lysis of the human fibrin plate frequently occurred at 3.125%.

It is evident that for estimation of plasminogen activator activity of human MNS the human plate is to be preferred as it is more sensitive than the bovine and there is no species difference between the substrate and the test salivas.

Conclusions:

1./

1. SHFP would be used for the estimation of plasminogen activator activity in human saliva.
2. SHFP could detect as little as 0.31 Ploug units/ml. urokinase after 19 hours incubation at $37^{\circ}C$.
3. SBFP could detect as little as 0.62 Ploug units/ml. urokinase after 19 hours incubation at $37^{\circ}C$.

Experiment 3

Object: To assess the inter-plate variation in sensitivity and its correction.

Method:

Positive and negative controls were set up with every SHFP and SBFP used. All fibrin plates were set up in one of three ways.

1. In which strong plasminogen activator activity was anticipated, e.g. Mixed native saliva. Fig. 2.
2. In which weak plasminogen activator activity was anticipated, e.g. Parotid saliva. Fig. 3.
3. "Screening". When large numbers of solutions were to be tested for activity and only a few were expected to be positive (e.g. Sephadex G200 fractions of salivary supernatant), plates as in Fig. 4 were set up, and a test solution showing evidence of lysis after 19 hours incubation was then set up again as in either 1 or 2 above, having been stored overnight at $4^{o}C$.

The areas lysed by urokinase in each part of every experiment were averaged and the result recorded as the "Urokinase Value" for that part of the experiment. Any experiment in which the tris buffer negative controls came up positive was considered totally invalid and the experiment was repeated. Many batches of bovine and human fibrinogen were used during the experimental work, but the urokinase control solutions were all derived from the one original solution prepared in October 1972 and deep frozen in 1 ml. aliquots at $-40^{o}C$.

When the fibrin plate experiments were completed, the recorded "Urokinase Values" for both human and bovine plates were collected and tabulated. Using a Hewlett-Packard HP.45 calculator, the mean/

FIGURE 2 TEST PLATE CONTROL PLATE

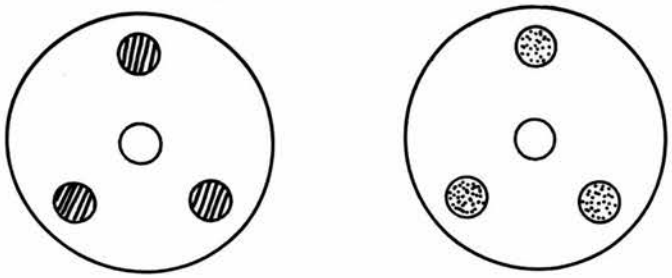


FIGURE 3

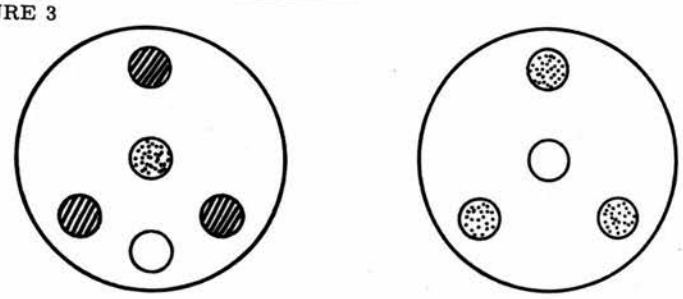
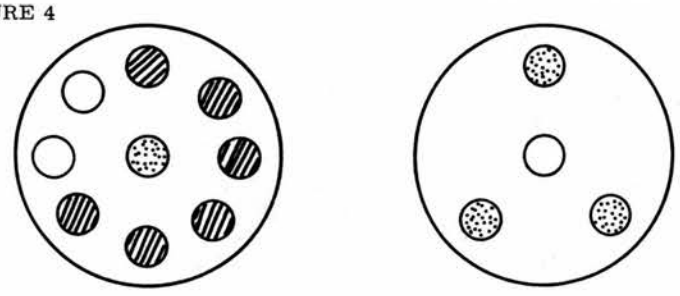


FIGURE 4



KEY

TEST SOLUTION

POSITIVE CONTROL. (Urokinase, 5 Ploug units/ml.)

NEGATIVE CONTROL. (Tris buffer. pH 7.8, 0.15 M)

All drop volumes were 0.03 ml.

mean and standard deviation for the areas lysed upon human and bovine plates by 0.03 ml. of urokinase 5 Ploug units/ml. were calculated.

Results:

These are recorded in Tables 4 and 5 (Appendix 1).

Summary of Results:

SHFP	Total number of "Urokinase Values" recorded	850
	Mean area of lysis	227 sq. mm.
	Standard deviation	57.89 (58)
SBFP	Total number of "Urokinase Values" recorded	1000
	Mean area of lysis	195 sq. mm.
	Standard deviation	55

All experimental results were now, retrospectively, corrected to allow for the variation in sensitivity between different batches of human and bovine fibrinogen. The correction is most simply explained by an example.

Test Solution. Average of three areas of lysis.	225 sq. mm.
Urokinase control. Average area of lysis.	289 sq. mm.
"Urokinase Value" upon SHFP.	227 sq. mm.

Therefore corrected value for test solution is $\frac{225 \times 227}{289}$

$= 176.73 = 177$ sq. mm.

Exactly the same procedure applies to bovine plates but using the bovine "Urokinase Value" of 195 sq. mm.

The results recorded in the experiments that follow are corrected values.

Experiment 4

Object: To demonstrate that the action of MNS upon SHFP is one of plasminogen activation and not non-specific proteolysis.

Principle:

Epsilon-amino-n-caproic acid (eACA) is a specific, potent, synthetic competitive inhibitor of plasminogen activation (Alkjaersig et al, 1959). Significant competitive inhibition of activation of human and bovine plasminogen by streptokinase, urokinase and tissue activator is seen at eACA concentrations of 10^{-4}M and higher, and at concentrations above 5×10^{-2}M eACA is a non-competitive inhibitor of plasmin and trypsin (McNicol and Douglas, 1964). At a concentration of 3×10^{-1}M, eACA non-competitively inhibits the activation of plasminogen by trypsin (Alkjaersig et al, 1959) and at concentrations of about 10^{-2}M inhibits pepsin (McNicol, 1964). If MNS has plasminogen activating activity then this activity will be significantly blocked by eACA at concentrations greater than 10^{-4}M but if the action is one of non-specific proteolysis, the fibrinolytic activity of the MNS will be unaffected.

Materials:

1. MNS. Using the technique of stimulated flow (Methodology, p XXVI) twenty samples of MNS, each not less than 10 ml. were collected from ten males and ten females. All were healthy dentates aged between 18 and 25 years, none of them were taking any drugs or showed any clinical evidence of gingivitis. This latter phrase means throughout this thesis, that any person with limited oedematous gingivitis (Code 1, Macphee and Cowley, 1969) or worse was not accepted as an experimental subject. Each sample was collected between 14.00/

14.00 and 14.30 hours and stored at 4°C until used.

2. Epsilon-amino-n-caproic acid (eACA) supplied by BDH Chemicals Ltd.

3. Human fibrinogen, thrombin, calcium chloride and tris buffer, as described for the preparation of SHFP. (Methodology, p.I)

4. Urokinase. 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.

Method:

Preparation of eACA solutions.

A. A molar concentration of eACA in tris buffer was prepared. From this were prepared the following solutions - M/2, M/4, M/8, M/16 and M/32.

B. An M/10 concentration of eACA was also prepared. From this were prepared the following solutions - M/20, M/40, M/80, M/160 and M/320.

Preparation of the plates. SHFP were prepared as usual, but incorporating in addition eACA in the following manner.

Plate 1. 0.4 ml. of M eACA. In a 10 ml. plate, this gives an end conc. of M/25.

Plate 2. 0.2 ml. of M eACA. In a 10 ml. plate, this gives an end conc. of M/50.

Plate 3. 0.1 ml. of M eACA. In a 10 ml. plate, this gives an end conc. of M/100.

Plate 4. 0.1 ml. of M/2. In a 10 ml. plate, this gives an end conc. of M/200.

This procedure was continued, adding only a 0.1 ml. volume of eACA each time until the complete range of eACA plates had been prepared.

In/

In order of dilution, the full range of dilutions was :

eACA end concentrations : M/25, M/50, M/100, M/200, M/400, M/800, M/1000, M/1600, M/2000, M/3200, M/4000, M/8000, M/16,000 and M/32,000.

Into the final plate was put 0.1 ml. of tris buffer.

Each of these fifteen plates was prepared in duplicate. Upon one set was placed MNS, and upon the other, urokinase. Each test solution was plated in triplicate (3 x 0.03 ml.) with a tris buffer negative control on each plate and a single urokinase drop on each MNS plate. After 19 hours incubation at 37°C the plates were read. The whole experiment was repeated twenty times with ten male and ten female MNS samples.

Results:

These are recorded in Tables 6 and 7 (Appendix 1) and on Graph 4.

Summary of Results:

The mean value and the standard deviation of the average areas of lysis in sq. mm. produced by 3 x 0.03 ml. of MNS (20 samples) and urokinase (20 tests) upon SHFP incorporating eACA at defined concentrations, after 19 hours incubation at 37°C.

eACA Concentration	MNS		Urokinase	
	Mean	S.D.	Mean	S.D.
M/25	0	0	0	0
M/50	0	0	0	0
M/100	0	0	*	*
M/200	0	0	52	53
M/400	0	0	107	18
M/800	0	0	141	40
M/1000/				

eACA Concentration	MNS Mean	MNS S.D.	Urokinase Mean	Urokinase S.D.
M/1000	0	0	149	28
M/1600	0	0	151	31
M/2000	32	45	160	27
M/3200	44	51	170	29
M/4000	58	71	173	24
M/8000	106	57	197	42
M/16000	128	45	228	31
M/32000	132	46	260	33
-ve eACA	150	54	266	35

*Probable lysis

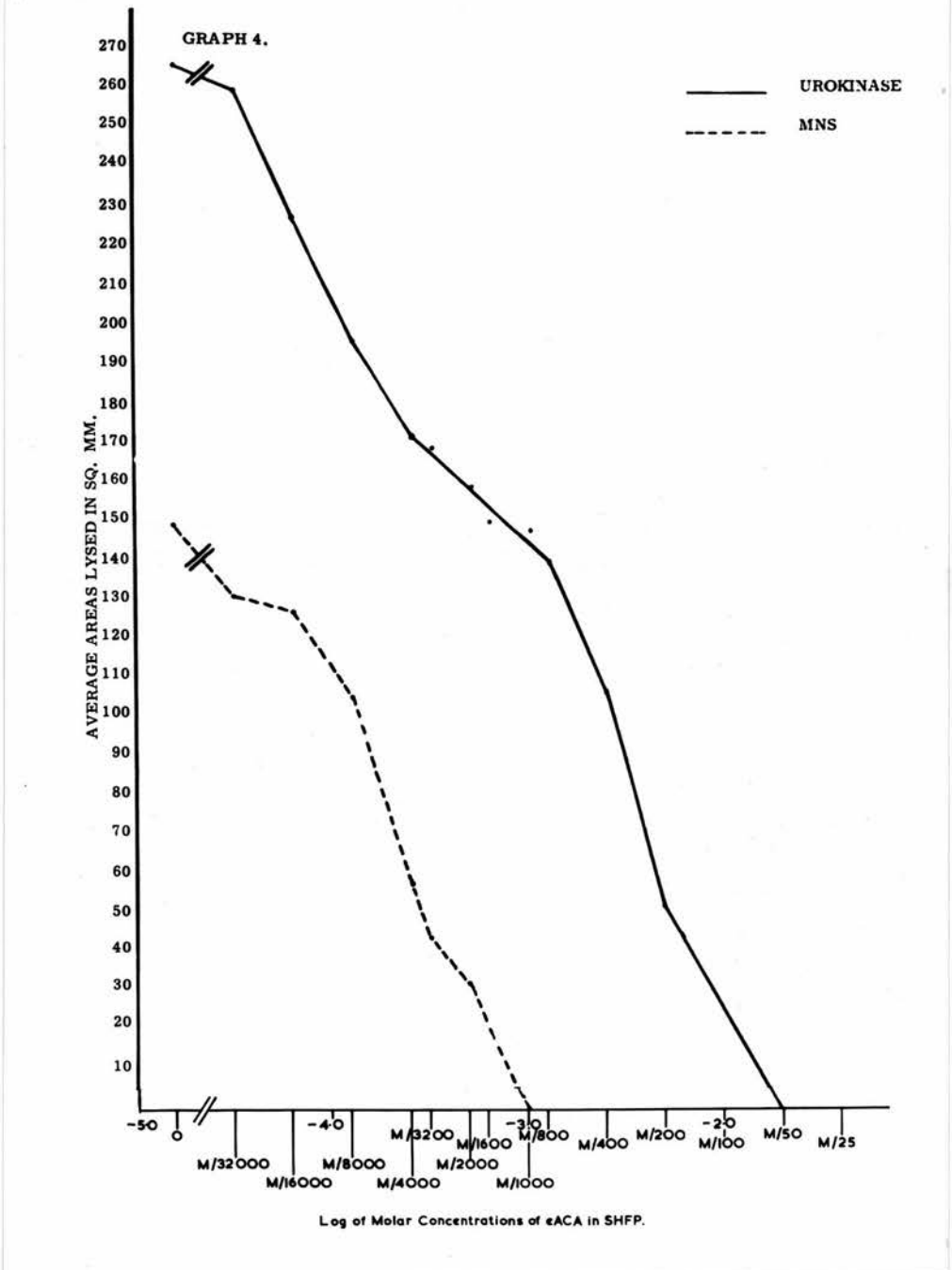
The negative control solution did not produce lysis.

Discussion:

The small number of subjects is justified since previous workers have produced strong evidence to show that MNS has fibrinolytic activity (Albrechtsen and Thaysen, 1955: Taylor et al, 1963: Schulte and Gewalt, 1964: Dolci, 1965: Nitta et al, 1967: Tortelli, 1967: and Wedgwood, 1970).

The nature of the experiment is justified since MNS does have some proteolytic activity (Willstatter et al, 1929: Weinmann, 1934: and Chauncey , 1961) and the fibrin plate has been used to determine non-specific proteolytic activity (Astrup and Alkjaersig, 1952: Brackman, 1967: and Haverkate and Traaf, 1974). The fibrin plate has not been used to measure non-specific proteolytic activity in MNS as far as the writer can ascertain.

These results suggest that the fibrinolytic activity of human MNS that is detectable upon a human fibrin plate is due to plasminogen activation/



Effect of eACA in SHFP upon the fibrinolytic activity of urokinase and MNS after 19 hours incubation at 37^{o}C.

activation and hence the presence of plasminogen activator(s) in human MNS.

Conclusions:

1. Human MNS has fibrinolytic activity.
2. Within the limitations of the fibrin plate technique, the fibrinolytic activity appears to be brought about by the specific activation of plasminogen contained in human fibrin plates and therefore activator(s) of plasminogen are present in MNS.
3. The system is insensitive to the action of unconcentrated non-specific proteolytic enzymes in MNS.

Experiment 5

Object: To obtain an estimate of the normal range of plasminogen activator activity in human mixed native saliva.

Materials:

1. MNS samples. These were collected by minimal stimulation from fifty male and fifty female healthy subjects aged 18 - 30 who were not taking drugs and who had no clinical evidence of gingivitis.
2. SHFP. (Methodology , p I).
3. Tris buffer. Negative control. pH 7.8, 0.15M.
4. Urokinase. Positive control in tris buffer, pH 7.8, 0.15M, 5 Ploug units/ml.

Method:

MNS was collected between 14.00 and 15.00 hours and after being vortex stirred, each sample was plated in triplicate upon SHFP. Control solutions were set up as described in Experiment 3, Fig. 2. The drop volume used throughout was 0.03 ml. The plates were incubated at 37^{o}C for 19 hours.

The experiment was repeated until MNS from 100 subjects had been examined.

Results:

These are recorded in Tables 8 and 9 (Appendix I).

Summary of Results:

Average areas of lysis in sq. mm. produced by 3 x 0.03 ml. of MNS upon/

upon SHFP after 19 hours incubation at 37°C.

	Male (50)	Female (50)	Total (100)
Mean	197	216	206
S.D.	100	109	104

There is no significant difference between the values obtained for male subjects and the values obtained for female subjects (t - test: t = 0.9).

Conclusions:

1. All MNS samples examined had plasminogen activator activity as measured upon SHFP.

2. The range of activity is very considerable and hence in every experiment in which an alteration to MNS is made (e.g. freeze-thawing, centrifugation, pH etc.) a control sample of MNS must always be set up upon SHFP in order to provide a base line from which to work and against which to measure the effect of experimental procedures.

3. There is no significant difference in plasminogen activator activity of MNS between males and females.

Experiment 6

Object: To determine whether or not the plasminogen activator activity of MNS is stable under Laboratory conditions.

Comment: The temperatures at which stability was assessed were : -40°C, 4°C, Room Temperature (RT) and 37°C.

1. -40°C

Collecting saliva is a time-consuming process and some experiments involve the use of very large quantities of saliva (e.g. analysis using a Sephadex column) and a "bank" would be extremely useful. The disadvantage of storing saliva at -40°C is the possible destruction of some of the organic constituents, in particular leukocytes and epithelial cells which may contain an activator which might be released on thawing and therefore give an artificially high reading for the plasminogen activating activity of MNS. It is possible, however, that only repeated freeze-thawing would achieve that result and that a single freeze, as with plasminogen and the commercially available fibrinogen solutions, would have no significant effect.

2. 4°C

This temperature has been used by many workers for keeping biological materials for a short duration prior to examination or analysis. The refrigerator in which MNS was placed was adjusted to 4°C and the difference between the top and the bottom of the refrigerator was not detectable with a 0 - 100°C thermometer.

3. Room Temperature

This was the most variable temperature. The range was 7 - 21°C

over/

over 24 hours on some days although the day time working conditions were maintained between 18° and 20°C as far as possible.

4. 37°C

Normal body temperature. The temperature of both incubators (LTE Water Jacket Type F Mark 2) was maintained between 37°C and 37.5°C.

The stability experiments carried out were as follows :-

1. Short Term Stability.
   - A. Period - 0 - 90 minutes.
   - B. Period - 0 - 180 minutes.
   - C. Period - 0 - 360 minutes.
2. Medium Term Stability. Period - 0 - 96 hours.
3. Long Term Stability.
   - A. Period - 0 - 120 hours.
   - B. Period - 0 - 12 months.

Materials:

1. Saliva. MNS was collected from healthy subjects aged 18 - 28, who were taking no drugs and who had no clinical evidence of gingivitis. The collection was as for stimulated MNS. (Methodology, P. XXVI).
2. SHFP. (Methodology , p I).
3. Positive and Negative controls as in Experiment 3, Fig. 2.

Stability Experiments.

1. Short Term Stability

1A. Short Term Stability: Period 0 - 90 minutes.

Subjects - Six. Three male and three female.
Temperatures - 4°C and 37°C.
Period - 0 - 90 minutes. Sampling at 10 minute intervals.
Procedure :-

MNS was collected from six subjects between 14.00 and 14.30 hours. Volume collected was 3.5 - 5.0 ml. by stimulated flow, per person. Immediately following collection, a salivary sample was vortex stirred and then 3 x 0.03 ml. drops were at once applied to a freshly made SHFP. This plate was put in a 37°C. incubator and the time recorded. The remainder of the sample was divided into equal aliquots, one of which was placed in an incubator at 37°C. and the other in the refrigerator at 4°C.

At ten minute intervals 3 x 0.03 ml. samples were taken from each aliquot (re-stirred) and plated upon SHFP all of which were incubated for 19 hours at 37°C.

A separate plate was used for each 10 minute sample in order that all plates could incubate for the same time. Upon each plate was set up a negative control, and control plates as described in Experiment 3, Fig. 2 were also set up. Samples were taken up to 90 minutes and each was read after 19 hours incubation at 37°C.

Results : These are recorded in Tables 10 and 11. (Appendix I).

Stability Experiment

1B. Short Term Stability: Period 0 - 180 minutes.

Subjects - Seven. Four male and three female.
Temperatures - 4°C, 37°C and RT.
Period - 0 - 180 minutes. Sample testing at 30 minute intervals.
Procedure :-

The procedure was similar to Experiment 1A except that the samples collected were divided into three aliquots, the third being set up at RT. In place of ten minute sampling, an interval of 30 minutes was adopted.

Results : These are recorded in Tables 12, 13 and 14. (Appendix I).

1C. Short Term Stability. Period 0 - 360 minutes.

Subjects - Five. Two male and three female.
Temperatures - 4°C. and 37°C.
Period - 0 - 360 minutes. Sample testing at 30 minute intervals.
Procedure :-

As above except aliquots were divided into two, 4°C. and 37°C. Incubation was 19 hours as before for each sample.

Results : These are recorded in Tables 15 and 16. (Appendix I).

Summary of Results

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. of MNS upon SHFP after 19 hours incubation at 37°C.

1A./

1A. Time Stored before Incubation

No. of Subjects		Minutes 0	30	60	90	
18	Mean	205	195	194	193	Temp. 4°C.
	S.D.	55	53	56	57	
18	Mean	205	191	184	179	Temp. 37°C.
	S.D.	55	49	48	46	

1B. Time Stored before Incubation

No. of Subjects		Minutes 0	60	120	180	
12	Mean	204	202	197	184	Temp. 4°C.
	S. D.	43	49	48	50	
12	Mean	204	183	163	141	Temp. 37°C.
	S.D.	43	40	31	32	

1C. Time Stored before Incubation

No. of Subjects		Minutes 0	120	240	360	
5	Mean	219	208	206	199	Temp. 4°C.
	S.D.	49	55	50	42	
5	Mean	219	170	137	108	Temp. 37°C.
	S.D.	49	34	37	22	

Stability Experiment

2. Medium Term Stability. Period 0 - 96 hours.

Subjects - Twenty. Ten male and ten female.

Temperature - $4^{o}C$, RT and $37^{o}C$.

Period - 0 - 96 hours. Sample testing at 24 hourly intervals.

Bacteriostat. Crystamycin (Glaxo Laboratories Ltd.) 400 mg./ml. in tris buffer.

Procedure :

Subjects presented themselves at the laboratory between 14.00 and 14.30 hours in groups of four. Stimulated MNS was collected (Methodology, p.XXVI) as a minimum of 12 mls. was required from each subject. Each sample collected was divided into six aliquots labelled A, A': B, B' and C, C'. 0.03 ml. of Crystamycin (0.6 mg./ml. end concentration) was placed in tubes A', B' and C'. A similar volume of tris buffer was added to A, B and C. From each of the 24 aliquots thus obtained was removed, after vortex stirring, three drops (0.03 ml.) of MNS and plated upon SHFP. Urokinase and tris buffer controls were set up as in Experiment 3, Fig. 2. These plates were incubated at $37^{o}C$ for 19 hours.

The aliquots were now stored at the defined temperatures, aliquots A and A' at $4^{o}C$, B and B' at RT (i.e. on a work bench away from direct sunlight and space heaters) and C and C' at $37^{o}C$ for 24 hours. After 24 hours incubation, the aliquots were re-sampled and plated upon fresh SHFP as before. The whole process was repeated until samples had been collected following 48, 72 and 96 hours storage.

The/

The experiment was repeated until MNS from all 20 subjects had been examined.

Results : These are recorded in Tables 17 - 22 (Appendix I).

Summary of Results

Medium Term Stability Studies at 4°C, RT and 37°C. Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of MNS upon SHFP after 19 hours incubation at 37°C.

Temp. of Stored MNS	Subjects (20)	Age of Samples 0	24	48	72	96 hours
	+ve Bact. *					
	Mean	237	227	222	209	202
	S.D.	43	49	48	42	40
4°C.						
	-ve Bact.					
	Mean	239	220	215	205	198
	S.D.	43	50	50	43	44
	+ve Bact.					
	Mean	237	216	188	171	152
	S.D.	43	63	49	51	39
R.T.	-ve Bact.					
	Mean	239	214	192	172	156
	S.D.	43	65	64	43	35
	+ve Bact.					
	Mean	237	90	29	0	0
	S.D.	43	91	55	0	0
37°C.	-ve Bact.					
	Mean	239	88	0	0	0
	S.D.	43	93	0	0	0

* Bact. With or without Bacteriostat.

Stability Experiment

3. Long Term Stability.
   A. Period 0 - 120 hours.
   B. Period 0 - 12 months.

Experiment A

Subjects - Twenty four. Twelve male and twelve female.

Temperature - 40°C.

Period - 0 - 120 Hours.

Procedure :-

Subjects presented themselves at the laboratory between 14.00 and 14.30 hours in groups of four. Stimulated MNS was collected (Methodology, p.XXVI) as a minimum of 12 mls. was required from each subject. Each sample was vortex stirred and then divided into six 2 ml. aliquots. Of these aliquots, five were immediately deep frozen at -40°C. From the remaining aliquots for each subject, 3 x 0.06 ml. volumes were removed and plated on SHFP. The plates were then placed upon level tables in the incubator and incubated for 19 hours at 37°C. Negative and positive controls were set up in accordance with the method described in Experiment 3, Fig. 2.

At 19 hours, these plates were read and the result recorded as the plasminogen activator activity of fresh MNS. At 24 hours, a single aliquot for each subject was removed from the deep freeze and allowed to thaw at room temperature. Having thawed the aliquots were vortex stirred and then plated upon fresh SHFP as above. The whole process was repeated at 24 hourly intervals, the last aliquot having been deep frozen for 120 hours. The experiment was repeated until twenty-four different salivas had been examined.

Results:

These are recorded in Table 23 (Appendix I).

Experiment B

Subjects - Twenty. Ten males and ten females.

Temperature - -40°C.

Period - 0 - 12 months.

Procedure:

Subjects presented themselves at the laboratory between 14.00 and 14.30 hours in groups of four. Stimulated MNS was collected (Methodology, pXXVI) as a minimum of 14 mls. was required from each subject. Each sample was vortex stirred and then divided into 13 x 1 ml. aliquots. Of these aliquots, 12 were immediately deep frozen at - 40°C. From the remaining aliquots for each subject, 3 x 0.06 ml. volumes were removed and plated on SHFP. The plates were then placed upon level tables in the incubator and incubated for 19 hours at 37°C. Negative and positive controls were set up in accordance with the method described in Experiment 3, Fig. 2.

At 19 hours, these plates were read and the result recorded as the plasminogen activator activity of fresh MNS. Thirty days later, a single aliquot for each subject was removed from the deep freeze and allowed to thaw at room temperature. Having thawed, the aliquots were vortex stirred and then plated upon SHFP (fresh) as above. The whole process was repeated at 30 day intervals and for a total of twenty subjects. The last aliquot had been frozen 360 days.

Results:

These are recorded in Table 24 (Appendix I).

Summary of Results for Long Term Stability experiments

A. Long Term Stability. Period 0 - 120 hours. Storage at -40°C.

Average/

A. Long Term Stability. Period 0 - 120 hours. Storage at -40°C.

Average areas of lysis (in sq. mm.) produced by 3 x 0.06 ml. drops of MNS upon SHFP after 19 hours incubation at 37°C.

Subjects	Age of Sample (in hours)					
(24)	0	24	48	72	96	120
Mean	243	225	222	228	232	242
S.D.	65	56	63	57	57	53

B. Long Term Stability. Period 0 - 12 months. Storage at -40°C.

Average areas of lysis (in sq. mm.) produced by 3 x 0.06 ml. drops of MNS upon SHFP after 19 hours incubation at 37°C.

Subjects	Age of Samples (in months)												
(20)	0	1	2	3	4	5	6	7	8	9	10	11	12
Mean	235	242	261	247	252	223	220	221	264	269	208	260	283
S.D.	67	69	71	85	95	74	59	52	74	87	60	86	67

Discussion:

The object of this experiment was to determine whether or not the plasminogen activator(s) in MNS was stable at defined temperatures, and if not, how quickly was the activity lost.

The Short Term experiments may be considered together. By combining the three experiments, there are readings for 18 subjects from zero to 90 minutes storage, at ten minute intervals, both at 4°C and 37°C. Over this period, there was no significant drop in activity at either temperature. At 4°C, $t = 0.62$ ($p < 0.5$) and at 37°C, $t = 1.49$ ($p < 0.2$). As the time of storage was increased, the pattern became clearer. After six hours stability had been retained at 4°C but there had been a significant loss of activity at 37°C, $t = 4.13$ ($p < .001$). The/

The short ten minute intervals suggested an initial loss of some activity within the first ten minutes, but this took place at 4°C as well as at 37°C, and was therefore probably unavoidable. In the event, this drop in activity was insignificant.

The experiment in which the effect of RT was assessed (0 - 180 minutes) was carried out first and no significant difference in activity was detectable over the first sixty minutes. As refrigerators were used to store solutions between tests and the centrifugations took place at 4°C, no bench procedure was foreseen that would take longer than one hour. Therefore no further short term experiments were conducted at RT.

Medium Term stability. It was expected that some procedures might take place over two or three days, which while not involving bench work at RT might necessitate apparatus and MNS being kept at RT (e.g. Sephadex Fractionation). Storage of several tubes of saliva, either to be able to set up all samples at the same time, or to pool them and so form a very large volume of saliva. could take place at 4°C if stability was assured. The use of 37°C in the stability experiment served to indicate the rate of decline of activity at incubation temperature. At this stage, it was not known whether or not bacteria played a significant role in the fibrinolytic activity of MNS. Storage of MNS at RT or 37°C and incubation of MNS upon fibrin plates might lead to increased bacterial populations and enhanced activity. The bactericidal broad spectrum antibiotic Crystamycin* was incorporated in duplicate test samples and comparison between MNS with and without a bacteriostat was made. Determination of the optimum strength of Crystamycin is explained in detail in the Bacteriological Experiments in Part IV.

The results of this part of the experiment are striking. At 4°C, there was very little loss of activity over a period of four days. There was no significant difference between the results obtained from samples containing/

* 300 mg. (500,000 units) Benzylpenicillin (sodium) B.P. plus 500 mg. Streptomycin sulphate B.P.

containing a bacteriostat and those without. Comparison of the 24 hour result with the six hour result obtained previously suggested that any loss occurred mainly in the first few hours. This may represent a highly labile substance in very small quantities. If plasminogen is found to be present in saliva. then it may be that this labile substance is plasmin. Although the results at RT showed a significant drop in activity, $t = 6.39$ ($p < .001$) with storage up to four days, there was still considerable activity and this indicated qualitative experiments extending over a period of a day or two may be successful. Again, there was no significant difference between the samples with bacteriostat present and those without. At 37°C, the findings were quite different. After 24 hours storage, only ten out of the twenty samples still showed activity and all were reduced in activity except one (Sample 2, Table 21) which was slightly increased. At 48 hours, all samples without a bacteriostat were negative although five with a bacteriostat were still showing evidence of activity. All activity was lost at 72 hours from each set of samples.

This loss of activity at 37°C may be due simply to the lability of the activator at this temperature, but it may also be due to the release of an inhibitor from the cellular components of the MNS. In vivo with a salivary clearance time of about twenty minutes, this would not be important to the fibrinolytic activity of the MNS.

It was apparent from these experiments that MNS can be stored overnight at 4°C and analytical work resumed the next day with little loss of activity. The long term stability experiments were designed to see whether or not samples of MNS could be stored for a very long time, if need be in order to build up a saliva 'bank'. The results indicated no significant loss of activity with storage at - 40°C.

Conclusions:

1./

Conclusions

1. The plasminogen activator(s) in MNS is stable in vitro at 4°C for up to at least 96 hours.

2. Storage at RT entails a significant loss of activity over this period but quite considerable activity remains nevertheless.

3. At 37°C, activity is considerably reduced after 24 hours and in the absence of a bacteriostat, is lost altogether at 48 hours. This is probably due to the lability of the activator(s) but may be due to the release of an inhibitor(s).

4. Long term storage of MNS at -40°C appears to entail no significant loss of activator activity.

Experiment 7

Object: To examine the intra-personal variation in the plasminogen activator activity of mixed native saliva.

Materials:

1. MNS. MNS was collected by minimal stimulation (Methodology, pXXV) from five male and five female healthy adults, aged 24 - 40, who had no clinical evidence of gingivitis. One male (I.N.) was a smoker (10 - 15 per day).
2. SHFP. (Methodology , p I).
3. Tris buffer. Negative control. pH 7.5, 0.15M.
4. Urokinase. Positive control in tris buffer, pH 7.5, 0.15M. 5 Ploug units/ml.

Method:

Collection of salivary samples was standardised and took place between 09.30 and 10.00 hours for the morning sample, and between 14.00 and 14.30 hours for the afternoon sample. These times were chosen to ensure an interval of at least one hour from the last meal.

Immediately after collection, each morning sample was stored at $4^{o}C$. After the afternoon collection, all samples (a.m. and p.m.) were vortex stirred and then individually plated (3 x 0.03 ml.) upon SHFP and controlled as described in Experiment 3, Fig. 2. The plates were then incubated for 19 hours at $37^{o}C$. The procedure was repeated for twenty consecutive working days to give a total of forty readings per person.

Results:

These/

Summary of Results

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of MNS upon SHFP after 19 hours incubation at 37°C.

	A.M.			P.M.			Total		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
Male									
J.M.	122	22	100-167	119	15	103-153	121	19	100-167
J.S.	98	13	70-117	105	17	70-128	102	15	70-128
H.M.	194	31	140-256	208	28	152-256	201	30	140-256
I.N.	118	22	100-202	117	17	100-161	117	20	100-202
P.H.	149	20	121-205	147	15	121-178	148	18	121-205
Female									
L.L.	107	9	93-125	110	9	103-141	108	9	93-141
E.R.	113	18	97-161	106	9	97-129	109	14	97-161
D.W.	115	29	93-197	122	20	100-161	119	14	93-197
E.Mc.	138	46	100-305	137	31	103-226	138	39	100-305
S.W.	211	35	125-256	224	36	152-278	218	35	125-278

These are recorded in Tables 25 to 28 (Appendix I).

Summary of Results: (See facing page).

Discussion:

The range of readings per person was very considerable. The smallest range (L. L., a.m.) was 93 - 125 and the largest (E. Mc., a.m.) was 100 - 305. There was no significant difference between morning and afternoon activity. Given such a large range of activity for each individual, it is necessary on all occasions to sample and plate freshly collected MNS before any procedure (e.g. centrifugation, or alteration of pH) is carried out in order to obtain a base line of activity for that single experiment.

Conclusion:

The range of intra-personal activity is too great to allow a single figure to be used as 'normal' and hence as a base line for future experiments. A fresh base line must be obtained in every experiment for every salivary sample to be tested.

CHAPTER III

SUMMARY OF CONCLUSIONS TO PRELIMINARY EXPERIMENTS

1. Standard human and bovine fibrin plates would be incubated for 19 hours at 37°C.
2. Standard human fibrin plates would be used for the estimation of plasminogen activator activity in human mixed native, parotid and submandibular/sublingual saliva.
3. Standard human fibrin plates can detect as little as 6.25% of a 5 Ploug units/ml. urokinase solution. That is, 0.31 Ploug units/ml. after 19 hours incubationat 37°C.
4. Standard bovine fibrin plates can detect as little as 12.5% of a 5 Ploug units/ml. urokinase solution. That is, 0.62 Ploug units/ml. after 19 hours incubation at 37°C.
5. Urokinase values for 0.03 ml. of 5 Ploug units/ml. urokinase upon standard human and bovine fibrin plates have been established retrospectively. They are :-

   A. Urokinase value upon SHFP. 227 sq. mm. (227 ± 58. 850 tests).

   B. Urokinase value upon SBFP. 195 sq. mm. (195 ± 55. 1000 tests).

   The method by which these figures are used to "correct" results is described.
6. Human mixed native saliva has fibrinolytic activity.
7. Within the limitations of the fibrin plate technique, the fibrinolytic activity of MNS appears to be brought about by the specific activation of plasminogen contained in human fibrin plates and therefore an activator(s) of plasminogen is present in mixed native saliva.

8./

8. The standard human fibrin plate system is insensitive to the action of unconcentrated non-specific proteolytic enzymes in human mixed native saliva.
9. All mixed native saliva samples examined have plasminogen activator activity as measured upon standard human fibrin plates.
10. The inter-personal range of activity is considerable (206 ± 104 sq. mm.) and hence in every experiment in which an alteration to mixed native saliva is made a control, unaltered sample of mixed native saliva must always be set up in order to provide a base line from which to work and against which to measure the effect of experimental procedures.
11. There is no significant difference in plasminogen activator activity of mixed native saliva between male and female.
12. The plasminogen activator(s) in mixed native saliva is stable at $4^{\circ}C$ for up to at least 96 hours.
13. Storage of mixed native saliva at room temperature entails a significant loss of activity over 96 hours although considerable activator activity remains.
14. At $37^{\circ}C$ plasminogen activator activity in MNS is considerably reduced after 24 hours and in the absence of a bacteriostat is lost altogether at 48 hours. This is probably due to the lability of the activator(s) but may be due to the release of an inhibitor(s).
15. Long term storage of mixed native saliva at $-40^{\circ}C$ appears to entail no significant loss of activator activity.
16. The range of intra-personal plasminogen activator activity of MNS is too great to allow a single figure to be used as 'normal' for any one person and hence as a base line for future experiments. A fresh base line must be obtained in every experiment for every salivary sample to be tested.

PART III

INVESTIGATIONS INTO THE SOURCE OF PLASMINOGEN ACTIVATOR ACTIVITY IN HUMAN MIXED NATIVE SALIVA

Chapter 1. Experiments:

Experiment 1. Comparison of plasminogen activator activity in MNS , parotid and submandibular/sublingual salivas.

Experiment 2. Centrifugation of MNS and examination of the plasminogen activator activity of various supernatants.

Experiment 3. Comparison of the plasminogen activator activity of MNS , the supernatant of MNS , filtered MNS and the filtered supernatant of MNS.

Experiment 4. The production of a supernatant of MNS containing no cells or cell fragments and its examination for the presence of a soluble activator.

Experiment 5. Investigation of the plasminogen activator of washed resuspended salivary pellet.

Experiment 6. Examination of MNS for the presence of a soluble activator using gel chromatography (Sephadex G200).

Chapter 2. Discussion and Conclusions.

CHAPTER 1

Experiment 1

Object: To compare the plasminogen activator activity of MNS with that of parotid and submandibular/sublingual salivas from the same subjects.

Materials.

1. Parotid, submandibular/sublingual and minimally stimulated MNS (Methodology, p. XXIX, XXX and XXV) from 40 adults (20 male and 20 female) aged 20 - 35 years, all of whom were in good health, were dentates and had no clinical evidence of gingivitis.
2. SHFP (Methodology, p. I).
3. Controls. Urokinase, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
   Tris buffer, pH 7.8, 0.15M.

Method:

Salivary samples each about 1 ml., were collected at 14.00 hours and stored at 4^{o}C.

When the collection was completed, the MNS samples were vortex stirred and then plated (3 x 0.03 ml.) upon SHFP. Controls of urokinase and tris buffer were plated as described in Part II, Experiment 3, Fig. 2. The submandibular/sublingual salivas and the parotid salivas were also set up in triplicate, but the plating pattern and controls were those for a weak activator as described in Part II, Experiment 3, Fig. 3.

All the plates were then/

then incubated at 37°C and read after 19 hours.

Four subjects were sampled each day until the series was complete.

Results: These are recorded in Tables 29 and 30 (Appendix I).

Summary of Results:

		Lysis	Probable Lysis	No Lysis
MNS	Male	20	-	-
	Female	20	-	-
	Total	40	-	-
Parotid	Male	11	3	6
	Female	6	7	7
	Total	17	10	13
Submandibular/Sublingual	Male	0	4	16
	Female	3	3	14
	Total	3	7	30

Discussion:

Of the 17 specimens of parotid saliva which showed lysis, only 9 showed activity similar to the value recorded for MNS. However, under conditions of minimal stimulation, the approximate gland volume contribution to MNS expressed as a percentage is 66.6% submandibular and 33.3% parotid (Kerr, 1961). Thus activator activity contributed to MNS by parotid saliva would be diluted by submandibular saliva by a similar ratio. In only one instance of the parotid being active was the submandibular active also, and therefore not acting as a dilutant in respect of activity.

If the major part of the MNS activator activity was derived from the parotid/

parotid, there would be consistently higher readings than in the MNS provided the submandibular activity was zero or very low. The converse would also be true for activity derived from the submandibular gland. Neither of these two circumstances pertain.

Conclusion:

This experiment suggests that while not excluding the possibility of a plasminogen activator being secreted by the parotid or submandibular/sublingual glands, these glands are not the major source of the activator in MNS.

Experiment 2.

Comment:

If the activator activity is in solution, then following centrifugation, the supernatant should retain its activator activity and indeed it may even appear enhanced if the cellular component has no activity since approximately 10 - 15% of the drop volume of MNS would have been occupied by inert matter prior to centrifugation.

Object:

Experiment 2: Part A.

To examine the plasminogen activator activity of MNS and that of its supernatant following low and high speed centrifugation.

Experiment 2: Part B.

To examine the plasminogen activator activity of MNS and that of its supernatant following short successive episodes of low speed centrifugation and again after high speed centrifugation.

Part A

Materials.

1. Stimulated MNS (Methodology, p. XXVI) was collected from 40 adults (20 male and 20 female) aged 20 - 35 years, all of whom were in good health, were dentates and had no clinical evidence of gingivitis.

2. Controls. Urokinase, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15 M.

   Tris buffer, pH 7.8, 0.15M.

3./

3. SHFP (Methodology, p. I).

4. Centrifuge.
   1. A universal Junior IKS (Heraeus-Christ GmbH, West Germany).
   2. An MSE Superspeed 40.

Method:

The collection of MNS was begun at 14.00 hours and a minimum of 5 ml. was obtained from each subject. Following collection, each sample was vortex stirred and then 1 ml. of MNS was removed and stored at $4^{o}C$. The remaining 4 ml. of each MNS were then centrifuged at $4^{o}C$ for one hour at 4154 g. Thereafter, the supernatant was decanted from each tube and 1 ml. stored at $4^{o}C$. (The salivary pellet obtained after the low speed centrifugation was deep frozen at $-40^{o}C$). The remaining 2 - 3 ml. of supernatant were then centrifuged for thirty minutes at 35,664 g. at $4^{o}C$, after which 1 ml. of supernatant was removed. Thus for each of the eight subjects, there were now three 1 ml. aliquots: MNS, low speed supernatant and high speed supernatant. Each specimen was plated (3 x 0.03 ml.) upon SHFP and controlled with urokinase and tris buffer (Part II, Experiment 3, Fig. 2). All the specimens for the day were plated at the same time as there would have been minimal loss of activity at $4^{o}C$ (Part II) and incubated for 19 hours at $37^{o}C$.

Results:

These are recorded in Tables 31 and 32 (Appendix 1).

Part B

Materials

Twenty adults, 10 Male and 10 Female) took part in this experiment. In all other respects, the "Subjects and Materials" are described in Part/

Part A.

Method:

The collection of MNS was begun at 14.00 hours and a minimum of 5 ml. was collected from each subject. Each sample was vortex stirred and 1 ml. removed and stored at 4°C as a representative sample of each subject's MNS.

The remaining 4 ml. of each MNS were then centrifuged at 4154 g. at 4°C. At fifteen minute intervals, the centrifuge was stopped and 0.5 ml. of supernatant was removed from each tube, placed in a fresh tube and stored at 4°C. This procedure was continued for up to one hour. After one hour the remaining supernatant from each subject was decanted and centrifuged at 35,664 g. for thirty minutes, after which 1 ml. of the supernatant thus formed was removed from each tube. For each of the eight subjects, there were then six aliquots: MNS, low speed supernatant at 15, 30, 45 and 60 minutes, and high speed supernatant. Each specimen was plated (3 x 0.03 ml.) upon SHFP and controlled with urokinase and tris buffer (Part II, Experiment 3, Fig. 2). All the specimens for the day were plated at the same time, and read after 19 hours incubation at 37°C.

Results:

These are recorded in Table 33 (Appendix 1).

Summary of Results

Part A

MNS. All samples had plasminogen activator activity.

Low speed supernatant:	12 samples (7M, 5F)	Lysis
	11 samples (6M, 5F)	Probable Lysis
	17 samples (7M, 10F)	No Lysis
	40 Total	

High speed centrifugation: 4 samples Probable Lysis
36 samples No Lysis

There was no difference in activity which was caused by sex.

Part B

MNS. All samples had plasminogen activator activity.

Low speed supernatant:

15 minutes.	All lysis.	All 20 appear to have reduced activity.
30 minutes.	18 lysis.	
	2 probable lysis.	
45 minutes.	10 lysis.	
	8 probable lysis.	
	2 no lysis.	
60 minutes.	4 lysis.	
	6 probable lysis	
	10 no lysis.	

High speed supernatant: 5 probable lysis.
15 no lysis.

Discussion

This reduction in activator activity of supernatants with increased centrifugation strongly suggests either the absence of a soluble activator or its presence in very small quantities and that the major activator component resides in the salivary pellet. Furthermore, it appears that much of the activity can be removed from suspension at relatively low speed centrifugation which suggests whole cells and large cell fragments play an important role.

That 23 of the 40 specimens displayed evidence of activity in the low speed supernatant and that of these, only 4 retained that property after/

after high speed centrifugation, is indicative of small active particles in suspension.

This was substantiated in samples M4, F8 and 9, where the plasminogen activator activity was actually seen to increase after low speed centrifugation, but was lost after high speed centrifugation from samples M4 and F8 and vastly reduced in F9. It is possible that in these samples there were many cell fragments present relatively diluted by many inert cells in the MNS and consequently present in higher concentration in the low speed supernatant.

Conclusion:

1. MNS has plasminogen activator activity (Reaffirmed).
2. Activator activity appears to reside predominantly in the salivary pellet.
3. Activity appears to be associated with large cells and large cell fragments as well as with small cell fragments and possibly bacteria and leukocytes.
4. A soluble activator may exist but if it does, its activity at the concentration it appears to have in MNS is below the threshold of this system to detect in all but a very few samples. It would appear to contribute little plasminogen activator activity to whole MNS.

Experiment 3

Object: To compare the plasminogen activator activity of MNS, the supernatant of MNS, filtered MNS and the filtered supernatant of MNS.

Comment:

It is reasonable to attempt to confirm (or refute) the findings of one experiment by examining the same problem with a different technique. It was decided, therefore, to see whether or not activator activity in MNS could be reduced by filtration.

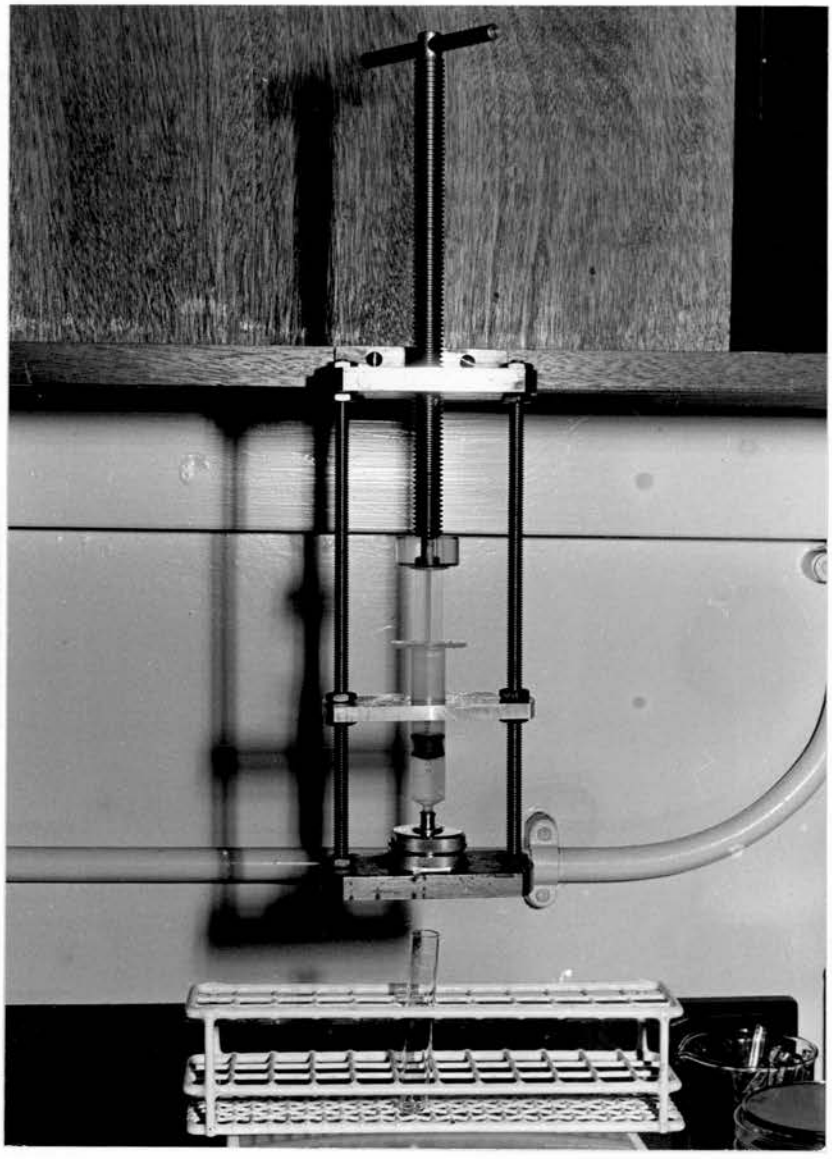
Subjects and Materials:

40 subjects (aged 19 - 26 years) all of whom were in good health, were dentates and had no clinical evidence of gingivitis.

1. Stimulated MNS (Methodology, p. XXVI) was collected from 40 subjects.
2. Centrifuge. A Universal Junior IKS, (Heraeus-Christ GmbH, West Germany).
3. Filters. Millipore, GS 0.22 micron 25 mm. Filter, supported in a Micro-syringe, Luer inlet, 25 mm. Millipore reference No. 30 025 00.
4. Micro-syringe Support and Screw Drive. Custom build in the Dental Hospital Technical Laboratory. Made in steel, it is illustrated in Fig. 5.
5. SHFP. Methodology, p. I).
6. Controls: Positive. Urokinase, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.

Negative /

FIGURE 5



Screw drive for filtering saliva through Millipore filters.

Negative. Tris buffer, pH 7.8, 0.15 M.

Method:

The collection of MNS was begun at 14.00 hours and a minimum of 10 ml. was collected from each subject.

The saliva was vortex stirred and a 1 ml. aliquot removed for plating on SHFP. Immediately after removing this aliquot, the remainder was divided into two equal volumes labelled 'A' and 'B'. Sample 'A' from each of the specimens collected was centrifuged at 4°C for 30 minutes at 4154 g. While samples 'A' were being centrifuged, samples 'B' were filtered through Millipore filters. The pore size (0.22 microns) is very small and the filtering of whole MNS had to be done very slowly in order not to distort the filter-supporting diaphragm. A steady heavy pressure was required and the steel screw drive was designed to provide such a force. The filtrates were stored at 4°C. When samples 'A' had finished centrifuging, 0.5 ml. was removed from each and stored at 4°C. The remainder was filtered in the same manner as sample 'B'. Thus was obtained a specimen of whole MNS, filtered MNS, supernatant of MNS and filtered supernatant of MNS for each subject. Upon SHFP, one for each specimen were placed three drops of the test solution and single drops of urokinase and tris buffer. The drop volume was 0.03 ml. Control plates as described in Part II (Experiment 3, Fig. 2) were also set up, and all plates were incubated at 37°C and read after 19 hours.

Results: These are recorded in Tables 34 and 35 (Appendix 1).

Summary of Results:

Effect of Samples upon SHFP

	Lysis	Probable Lysis	No Lysis

1./

1.	MNS	40	0	0
2.	MNS Filtrate	1	10	29
3.	MNS Supernatant	40 (reduced)	0	0
4.	MNS filtered Supernatant	19	7	14

There was no difference in activator activity that was caused by a difference in sex.

Discussion:

These results were consistent with Experiment 2 in suggesting either the absence of a soluble activator or its presence in very small amounts. The one active MNS filtrate may have been due to a faulty filtering technique allowing some cells through, or it may have represented some very small particles that were filtrable. This second suggestion appears probable in the light of the activity found in the filtrate of the supernatant of MNS. This filtrate had measurable lysis on a total of 19 occasions and probable lysis an additional seven times. The activity was always reduced relative to whole MNS and the supernatant of MNS.

The difference in activity between the filtrates was probably due to the occlusion of the tiny Millipore pores by constituents of the salivary pellet thus allowing only the smallest particles through. The filtrate of the supernatant of the MNS was easily produced which is indicative of much less blockage of the filter pores.

The two filtrates considered together suggest the absence of a soluble activator, but the presence of activator activity which is cell associated and which, in addition, is associated also with very small cell particles.

Conclusion:/

Conclusion:

The findings of Experiment 2 are confirmed using a different technique. The plasminogen activator activity of MNS appears to reside almost entirely in the pellet and to be associated with cells and cell particles.

Experiment 4

Object: To produce a supernatant of MNS that contains no cells or cell fragments and to examine it for the presence of a soluble activator.

Comment:

Experiments 1 - 3 taken together provide little evidence for the presence of a soluble activator in MNS. However, it can be argued that in Experiment 2 (A) four samples and in Experiment (B) five samples showed probable lysis after high speed centrifugation. In Experiment 3, after filtering MNS supernatant from forty subjects, nineteen displayed lysis and seven probable lysis.

There is no escaping the fact that 0.22 microns is a very small filter pore size and, despite the argument laid out in the discussion in Experiment 3, some doubt still remained as to the presence of a soluble activator. At least, it would appear that the filter, per se, did not inactivate the activator, a possibility to be considered had no activator activity appeared in the filtrate.

Centrifugation at very high speed can destroy cells and this could cause "false positives" by releasing into suspension, cell associated activators whose activity might be construed as being the activity of a soluble activator. Therefore one more filtering and centrifuging experiment was undertaken.

Materials:

1. Centrifuges. As described for Experiments 2 and 3.
2. Millipore filters. 0.22 micron pore size. Filtration method as described in Experiments 2 and 3.
3. SHFP (Methodology, p. 1).

4./

4. Minicon B15. A disposable microconcentrator supplied by Amicon Ltd., Buckinghamshire. The '15' indicates a molecular weight cut off at 15,000.

5. Controls. Positive. Urokinase, 5 Ploug units/ml. pH 7.8, 0.15M. Negative. Tris buffer, pH 7.8, 0.15M.

6. Subjects. Twenty students (10 Male, 10 Female) aged 19 - 26 years, all of whom were in good health and had no clinical evidence of gingivitis.

7. Collection of saliva. Stimulated mixed native, parotid and submandibular/sublingual saliva were collected in accordance with the methods described in Methodology, p. XXVI, XXIX & XXX.

Method:

MNS. A minimum of 10 - 12 ml. was collected and after vortex-stirring, 0.5 ml. was stored at $4^{o}C$.
The remaining MNS was centrifuged at 4154 g. at $4^{o}C$ for thirty minutes after which a further 0.5 ml. was removed and stored at $4^{o}C$. The remaining supernatant was filtered through an 0.22 micron Millipore filter. Following filtration, another 0.5 ml. aliquot was removed and stored at $4^{o}C$. The filtrate was then finally centrifuged at 35,664 g. for thirty minutes. From the supernatant generated, 0.5 ml. was removed and stored at $4^{o}C$. Finally, all but 0.5 ml. of the remaining supernatant was gently removed from the centrifuge tube to avoid disturbing any pellet that might have been present and 5 ml. was poured into one channel of a Minicon B15 where it was concentrated 25x at room temperature, a process taking about $1\frac{1}{2}$ hours. The remaining 0.5 ml. in the centrifuge tube was vortex stirred and, after making two smears for cytological examination, was stored at $4^{o}C$.

Therefore, for every sample of MNS, the following preparations were made./

made.

Whole MNS, low speed supernatant of MNS, filtered supernatant, high speed supernatant, concentrated high speed supernatant. In addition, a smear for cytology was prepared from the high speed pellet which itself was resuspended in 0.5 ml. of high speed supernatant.

Parotid and submandibular/sublingual saliva from the same subjects were subjected to a similar process except that the first low speed centrifugation was omitted and no smear was made of the high speed centrifugation 'pellet'. All the plates were set up at the same time and incubated for 19 hours at 37°C. Control plates as described in Part II, Expt. 3, Fig. 3 were also set up. The drop volume throughout was 0.03 ml.

Results:

These are recorded in Tables 36 - 39 (Appendix 1).

Summary of Results:

	Effect of samples upon SHFP		
	Lysis	Probable Lysis	No Lysis
A. MNS			
1. Whole MNS	20	0	0
2. MNS Supernatant (4154 g.)	16	2	2
3. Filtered Supernatant	4	7	9
4. No. 3, centrifuged at 35,664 g.	0	0	20
5. No. 4 conc. x 25	0	0	20

B./

B.	Parotid			
1.	Whole Parotid	6	6	8
2.	Filtered Parotid	1	2	17
3.	No. 2, centrifuged at 35, 644 g.	0	0	20
4.	No. 3 conc. x 25	0	0	20
C.	Submandibular/sublingual			
1.	Whole submandibular/ sublingual	0	4	16
2.	Filtered submandibular/ sublingual	0	0	20
3.	No. 2 centrifuged at 35, 644 g.	0	0	20
4.	No. 3 conc. x 25	0	0	20

Discussion:

The results obtained after filtering the MNS supernatant were lower than previously obtained and probably reflect an improved filtering technique. The complete absence of any detectable plasminogen activator activity following high speed centrifugation and concentration is very strong evidence to support the proposition that no soluble plasminogen activator is present in the supernatant of MNS, parotid or submandibular/sublingual saliva. The result was a little surprising as concentrating 25x might reasonably have brought about lysis due to protease activity in the MNS and then it would have been necessary to examine the concentrate on eACA plates. As it was, no activity could be detected.

The examination of the consistently active high speed pellets was very revealing. Not only were tiny cell fragments present, but also much larger fragments and intact desquamated epithelial cells that must have been forced round the edge of the Millipore filter. When the pellets/

pellets were examined by fibrinolytic autography (Methodology, p.V) some of the cells and fragments were shown to be active. (Fig. 6 - 9) This emphasized the incomplete nature of the filtering process. No criticism is being made of the filters themselves, but rather of the filter holders. It is apparent from these results that the filter supporting diaphragm and the two washers that support it must be regularly inspected. The results further illustrate the necessity to control such procedures by visual examination of the filtrate.

The finding of such large cell fragments in the filtrate must throw some suspicion on the findings of Experiment 3 where it was argued that many of the cell fragments must be very tiny indeed in order to pass through the filter pores. It was now apparent that this may not be the case and the previously recorded activity may well have been due to larger fragments passing around the filter when the supernatant was filtered and to even larger fragments preventing such a passage when whole MNS was filtered.

Nevertheless, the essential finding remains unaltered, namely that there appears to be no soluble plasminogen activator in MNS, parotid or submandibular/sublingual saliva and that the activator activity of MNS is cell and cell fragment associated.

Conclusions:

1. There is no plasminogen activator in solution in MNS, parotid or submandibular/sublingual saliva within the capacity of the fibrin plate technique to detect.

2. The plasminogen activator activity of MNS is cell and cell fragment associated.

FIG. 6

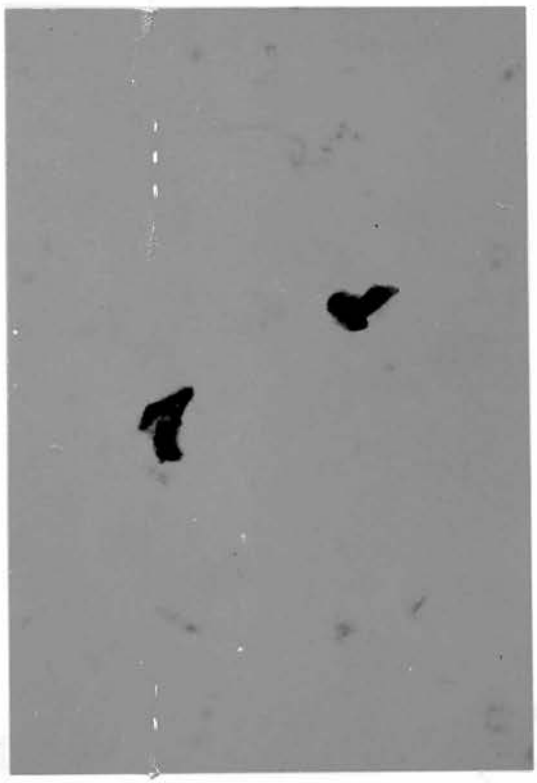
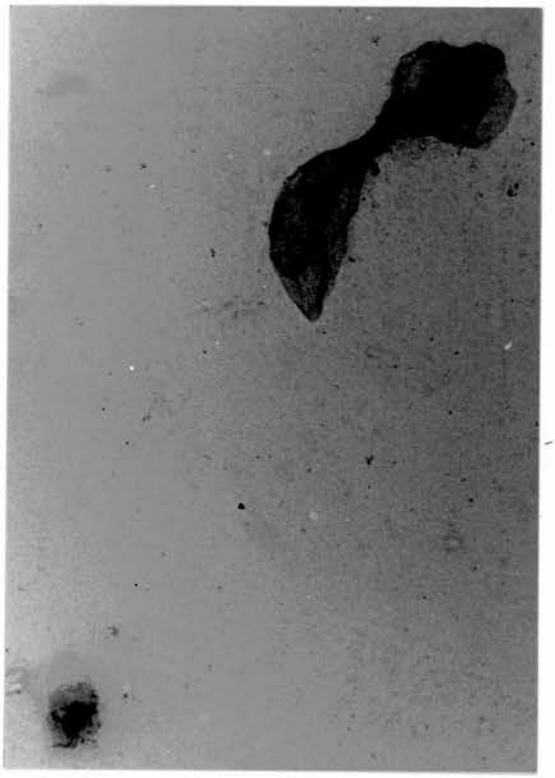


FIG. 7



FIGS. 6 and 7. Smears of high speed pellet following low speed centrifugation and filtering through 0.22 micron Millipore filter, showing nucleated squamous epithelial cells and cell fragments.
Stained with Papanicolaou. Mag. x 300.

Fig. 8.

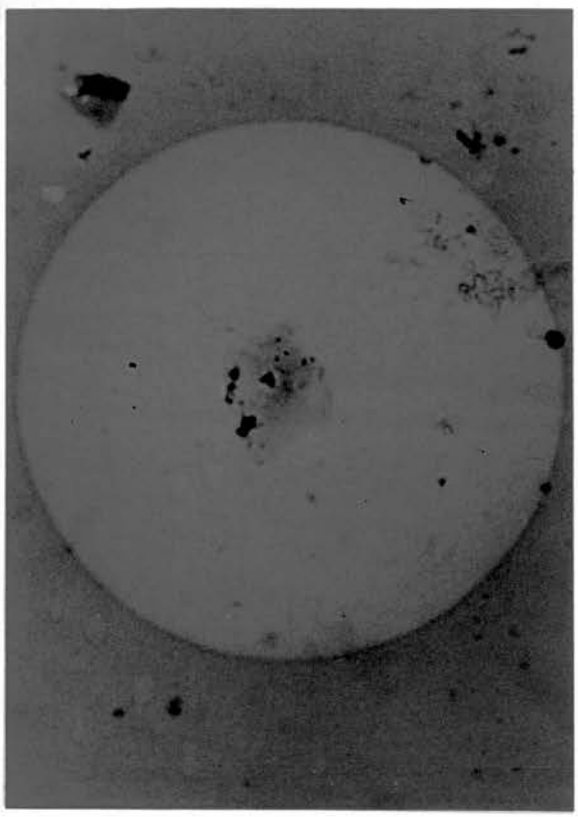
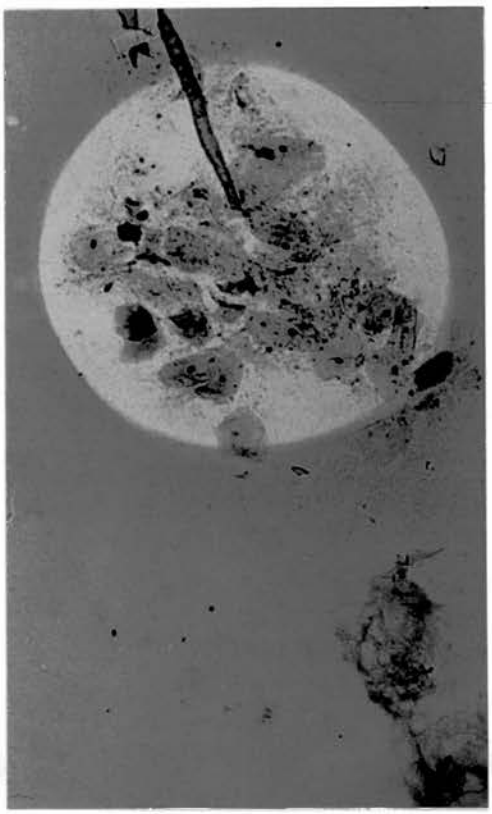


Fig. 9.



Figs. 8 & 9. Fibrinolytic autographs of smears of high speed pellet showing lysis related to epithelial cells and cell fragments. Human fibrin incubated overnight at room temperature followed by 1 hour at 37°C. Mag. Fig. 8. x 300. Fig. 9. x 120 .

Experiment 5

Object: To investigate plasminogen activator activity of washed resuspended salivary pellet.

Materials:

40 students (20 Male, 20 Female) aged 19 - 26 years, all of whom were in good health, were dentates and had no clinical evidence of gingivitis.

1. Stimulated MNS (Methodology, p. XXVI) was collected from 40 students.
2. Centrifuge. A Universal Junior IKS (Heraeus-Christ GmbH, West Germany).
3. SHFP (Methodology, p. I).
4. Controls. Urokinase, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
   Tris buffer, pH 7.8, 0.15M.
5. Tris buffer was also used as the washing medium for the pellet and also for its resuspension.

Method:

The collection of MNS was begun at 14.00 hours and a minimum of 5 ml. was collected from each subject.

From each sample was removed, 0.5 ml. which was then stored at 4^{o}C. The remaining 4.5 ml. of each sample was centrifuged for 30 minutes at 4154 g. From the supernatant thus formed, 0.5 ml. was removed and stored at 4^{o}C. The remaining supernatant was discarded.

The/

The salivary pellet of each specimen was washed by centrifugation (10 mins. x 4154 g.) in tris buffer four times. The pellet was then reconstituted to 4.5 ml. in tris buffer. A 0.5 ml. volume of the final washing, and 0.5 ml. of the resuspended pellet from each specimen were stored at 4°C. The washing procedure was repeated a further four times and samples of the final washing and resuspended pellet were again stored at 4°C.

Thus for each original specimen of MNS, there were now six aliquots to be tested upon SHFP.

These were :

1. Whole MNS.
2. Supernatant of MNS generated at 4154 g. in 30 minutes.
3. Supernatant of washed pellet after four washes.
4. Resuspended pellet after four washes.
5. Supernatant of washed pellet after eight washes.
6. Resuspended pellet after eight washes.

Upon SHFP were placed three drops of each test solution and single drops of urokinase and tris buffer. The drop volume was 0.03 ml. Control plates as described in Part II, Expt. 3, Fig. 3 were also set up. All the plates were set up at the same time, incubated at 37°C and read after 19 hours.

Results:

These are recorded in Tables 40 and 41 (Appendix 1).

Discussion:

Analysis of Results:

Test/

Test	Male	Female	Total
1. MNS. Lysis.	20	20	40
2. Sup. of MNS. Lysis.	20	20	40
3. Sup. of pellet after 4 washes:			
a) Lysis.	5	6	11
b) Probable Lysis.	8	3	11
c) No lysis.	7	11	18
4. Resuspended pellet after 4 washes:			
a) Lysis.	16	18	34
b) Probable Lysis.	3	2	5
c) No Lysis.	1	0	1
5. Sup. of pellet after 8 washes:			
a) Lysis.	0	0	0
b) Probable Lysis.	3	2	5
c) No Lysis.	17	18	35
6. Resuspended pellet after 8 washes:			
a) Lysis.	13	16	29
b) Probable Lysis.	5	3	8
c) No Lysis.	2	1	3

Tests 1 and 2 reaffirmed that MNS had activator activity and that the supernatant of MNS generated at low speed centrifugation was also active. Therefore, some activator activity resided either in solution and/or in small particles still in suspension. Test 3 indicates that 22 of the 40 samples still had evidence of activator activity in the supernatant.

Possible explanations :

1. A very strong soluble activator in the original test solution. Most improbable. The highest recording of lysis for supernatant was 312 (Female No. 2) and after washing the pellet, this was reduced to 'probable lysis' in the supernatant, but in eleven other instances following the same procedure, the reduction was very much less, /

less, and furthermore, the activity of the MNS supernatant in these eleven was less than that displayed by Female No. 2.

2. It was highly improbable that fresh activator had been released so quickly into solution from possible reservoirs (epithelial cells or leukocytes) as the stability studies recorded in Part II did not indicate anything of that nature nor have substances been used here in washing the pellet, such as potassium thiocyanate or detergent, which might have released an activator.

3. An explanation that is consistent with the findings is that the activity was associated with small particles, many of which would be in the pellet following the first centrifugation since the mucus present in MNS would bring many of them down. Following washing, however, the mucus became extremely dilute and would "release" many particles into suspension. The amount of mucus in MNS is very variable as is the number of cells and cell fragments and, therefore, it would be quite possible to obtain high readings of activity, by this hypothesis, until such time as the mucus is so dilute as to be incapable of trapping any small particles and all such particles are lost in the discarded washings.

Test 4. Thirty-nine of the samples showed evidence of lysis which strongly indicates activator activity retained in whole cells and/or large cell fragments brought down at low speed centrifugation. Test 5 showed only five specimens with activator activity still present in the supernatant after eight washes and the explanation offered above is applicable here also. Test 6. Thirty-seven of the test samples showed evidence of lysis and, although on scrutiny it will be seen that the lysis obtained was in most cases reduced, this could be due to loss of cells and of some of the larger fragments with washing. In retrospect, a cell count might have been useful but it is very difficult to carry out an accurate differential count in MNS due to the difficulty in obtaining a representative drop (the mucus makes even dispersion almost impossible) and the altered appearance of leukocytes.

Conclusion/

Conclusion:

The results are consistent with the findings obtained in Experiments 1 - 4. The activator activity appeared to be associated with whole cells and/or very large cell fragments, and to the cell particles described in Experiment 4. There was no evidence to suggest that a soluble activator plays any significant role in the MNS activator system.

Experiment 6

Object: To examine MNS for the presence of a soluble plasminogen activator using Sephadex Fractionation.

Comment:

In the course of examining MNS for the presence of plasminogen (Part V), pooled supernatant was, after concentration, fractioned on a G200 Sephadex column. The fractions were tested for plasminogen with streptokinase and tanned red cell haemagglutination inhibition immunoassay and the molecular weight range of the active fractions examined to ascertain whether or not they were in the same order of magnitude as might be expected from plasminogen. The full experimental details are described in Part V, Experiment 6.

Each fraction was also examined for activator activity by plating upon SHFP in the manner described in the preceding experiments.

The control solutions of urokinase and tris buffer were set up as described in Part II, Experiment 3, Fig. 4 and in addition 3 x 0.03 ml. of the eluant AG azide saline (Aronson-Gronwal). Both the tris buffer and the eluant proved negative.

Results:

No activator activity appeared in any fraction in the ten runs performed.

Discussion:

The importance of this experiment, in terms of activator activity, lay in the possibility that an activator and its inhibitor might be separated by/

by a molecular weight difference and some evidence of activator activity become apparent. No such evidence emerged. On two occasions all the fractions were individually concentrated 25x and replated upon SHFP but no activator activity was found. It cannot be concluded with absolute certainty that there is no soluble activator in the supernatant of MNS for at least two reasons.

1. As will be discussed in Part IV, 'Salivary Flora', there is evidence in the literature that proteolytic enzymes of bacterial origin are present in MNS. No evidence of such proteases was found in any of the fractions which suggests the system was insensitive to very weak proteases although, of course, their molecular weight may have been outwith that examined by a G200 column. Their presence would have been indicated by lysis of the SHFP and they would have been differentiated from plasminogen activators by applying them to SHFP incorporating eACA. It is, therefore, possible that a soluble plasminogen activator is present but it must be extremely weak.

2. Stability. In order to carry out this experiment, more than 100 ml. of MNS was required. This was collected in one day, pooled, centrifuged, and the supernatant concentrated to about 7 - 8 ml. using Polyethylene Glycol. From collecting the first MNS sample to starting the column run, a minimum of 48 hours elapsed. The run was then carried out at room temperature and the fractions, before being available for plating, had their optical density measured. At the very least, a total of 72 hours elapsed between collection and plating on SHFP and most of this at room temperature. In Part II, activity of MNS was shown to be still present at 72 hours, having been stored at room temperature, but it was reduced. It was known from the preceding experiments that if any soluble activator was present, it must be in small amounts and, therefore, any loss of activity during an experimental procedure might falsify the results. At the time of doing this experiment it was not possible to run the MNS at $4^{o}C$ but columns are/

are now available at this temperature and a further one or two runs at 4°C should be performed to examine the possibility of a heat labile soluble activator present in very small concentrations.

Conclusions:

Sephadex fractionation on a G200 column has failed to provide any evidence of a soluble activator in MNS supernatant.

CHAPTER 2

Discussion and Conclusions

From the evidence provided by centrifugation, filtration and gel chromatography, no soluble activator could be detected in natural or concentrated MNS using SHFP. The results showed that the activator was present in the salivary pellet, and suggested that it was a cell and cell fragment associated activator.

The conclusion that there is no detectable soluble activator within the sensitivity of this system is important because there is a tacit acceptance amongst reviewers of 'Fibrinolysis' that saliva (unqualified) has fibrinolytic activity (Macfarlane, 1964: McNicol and Douglas, 1972). If the activator is not in the secretions of the salivary glands, there is no reason to believe it is required to help maintain the patency of the salivary ducts; a function that has been suggested for urokinase in respect of the ureter (McNicol et al, 1961). The total absence of a soluble activator has not been established beyond all doubt but if it does exist, it must be very weak and, therefore, it was necessary to investigate systematically the contents of the salivary pellet to determine the major source of the plasminogen activator activity of MNS.

PART IV

THE PLASMINOGEN ACTIVATOR ACTIVITY OF THE SALIVARY PELLET

CHAPTER I

THE WHOLE PELLET

Comment:

The evidence presented in Part III strongly suggested the absence of a soluble plasminogen activator in mixed native, parotid and submandibular/sublingual saliva, and that the activator resided in the salivary pellet. Allowing the transient presence of bacterial pathogens and food debris, the pellet may be considered to comprise mucus, commensal bacteria, leukocytes, epithelial cells and cell fragments and these were systematically examined (Chapters 3 - 6)

Object: To examine whole salivary pellet for fibrinolytic activity using SHFP.

Materials:

MNS. MNS (Methodology, p. XXVI) was collected from 20 healthy subjects who had no evidence of gingivitis.

Salivary pellet. From each sample of MNS collected, 8 ml. was removed and centrifuged at 4154 g. for 30 minutes after which the supernatant was decanted and the pellet reconstituted in tris buffer to a volume of 1 ml.

SHFP. (Methodology, p. I)

Tris buffer. Negative control. pH 7.8, 0.15 M.

Urokinase. Positive control. 5 Ploug units/ml. pH 7.8, 0.15 M.

Method:

Each salivary pellet was serially diluted in tris buffer to provide concentrations of 100, 50, 25 and 12.5%. Every dilution was plated (3 x 0.03 ml.) upon SHFP and incubated for 19 hours at 37°C.

Controls/

Controls of urokinase and tris buffer were set up as described in Part II, Experiment 3, part 2.

Results:

These are recorded in Table 42, (Appendix 1).

Summary of Results:

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of salivary pellet upon SHFP after 19 hours incubation at 37°C.

	100%	50%	25%	12.5%
Mean (20)	170	133	102	*
S.D.	62	37	12	*

* Probable lysis with 5 specimens. Lysis in remainder.

Discussion:

Activity decreased with increased dilution of the pellet. As the supernatant was known to be inactive then this reduction in activity must have been caused by the dilution of either mucus or a cell associated activator. When the salivary pellet was reconstituted to the original volume of MNS (i.e. 12.5%) the areas of lysis on SHFP were less than was normally obtained with MNS. In Part III it was shown that some of the fibrinolytically active cellular material in MNS remains in the supernatant after centrifugation at 4154 g. for 30 minutes and so it was not expected that such reconstituted MNS would be as active as the "natural" MNS from which it was derived.

Conclusion:

Activator/

Activator activity is present in the salivary pellet and activity decreases with dilution of the pellet.

CHAPTER 2

DENSITY GRADIENT EXPERIMENTS

Object: To show whether or not plasminogen activator activity of salivary pellet can be separated out into a particular density fraction.

Materials:

1. Pellet. MNS was collected by stimulated flow (Methodology, p. XXVI) from persons with no clinical evidence of gingivitis. After centrifugation at 4154 g. for 30 minutes the supernatants were discarded and the pellets pooled.
2. Density gradient. Equal parts of sucrose (60% w/w) and Triosil 350, (Intra-venous contrast medium, Nyegaard & Co., Oils; 70% w/w) were mixed (this was called 60% w/w) and then diluted in saline to give 9 fractions of decreasing density, at 5% intervals from 60% - 20% (density 1.336 - 1.082).
3. SHFP. (Methodology, p. I)
4. Controls. Urokinase, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15 M.
   Tris buffer, pH 7.8, 0.15 M.
   Gradient medium.

Methods:

1 ml. of the pellet was vortex stirred with 1 ml. of the 20% fraction and the mixture overlaid on a gradient containing 1 ml. of each of the other density fractions. The whole was centrifuged for either 10, 20 or 30 minutes at 4154 g. at 4°C. The fractions were then removed and from each 3 x 0.03 ml. drops were plated upon SHFP with controls as described in Part 2, Experiment 3, Fig.3 and incubated/

Fig. 10.

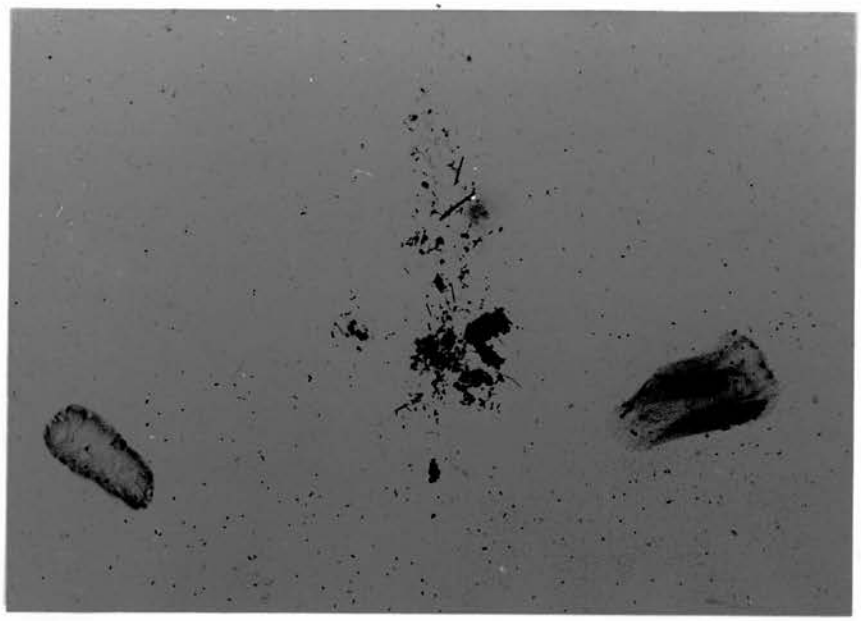


Fig. 11.

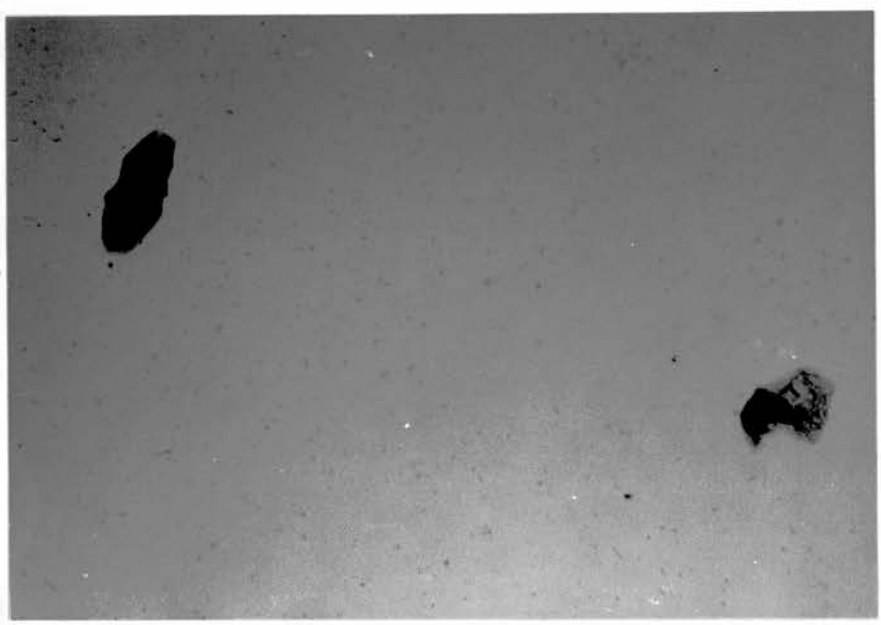


Fig. 10. Bacteria , nucleated squamous cell and anuclear cell in Fraction 1 (Density Gradient). Stained with Azure. Mag. x 300.

Fig. 11. Anuclear epithelial cell fragments in Fraction 1 (Density Gradient).
Stained with Azure. Mag. x 300.

Fig. 12.

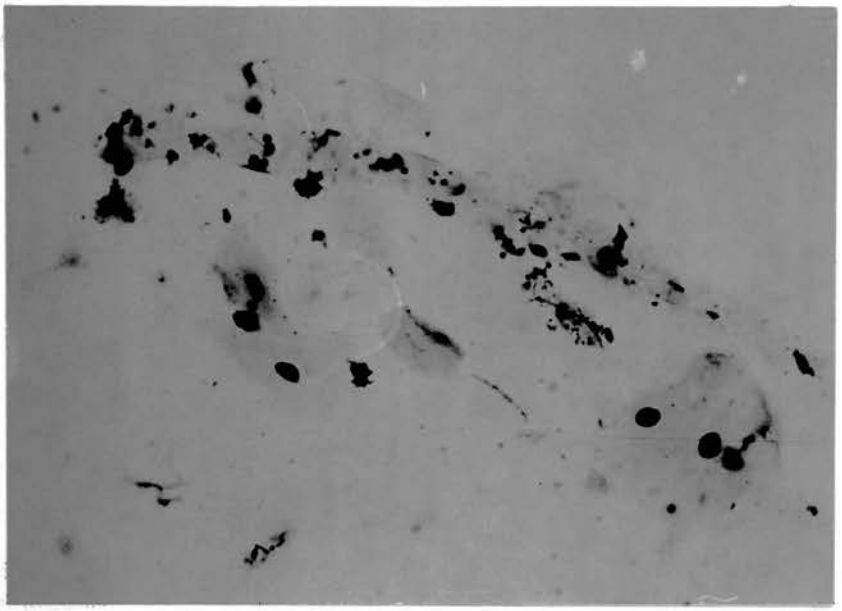
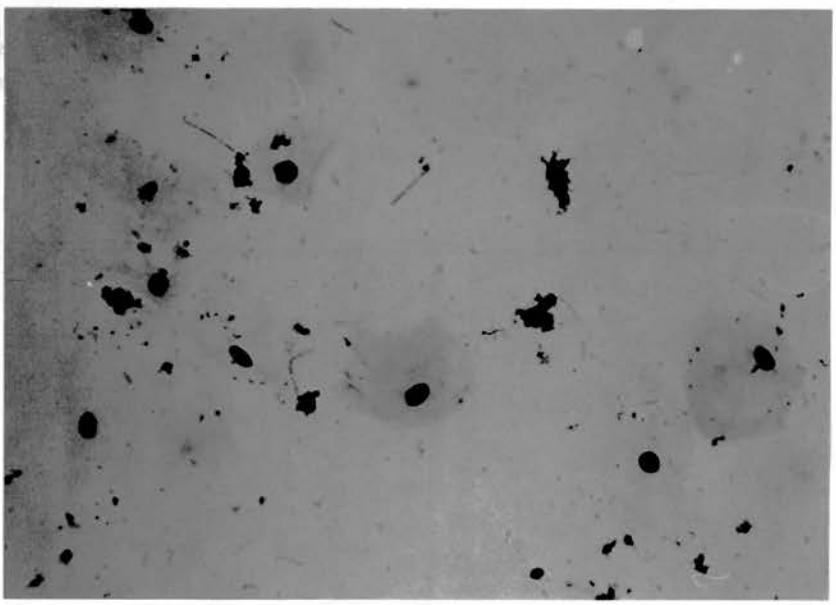


Fig. 13.



Figs. 12 & 13. Numerous nucleated epithelial cells and bacteria in Fraction 9 (Density Gradient). Stained with Azure. Mag. x 300.

Fig. 14.

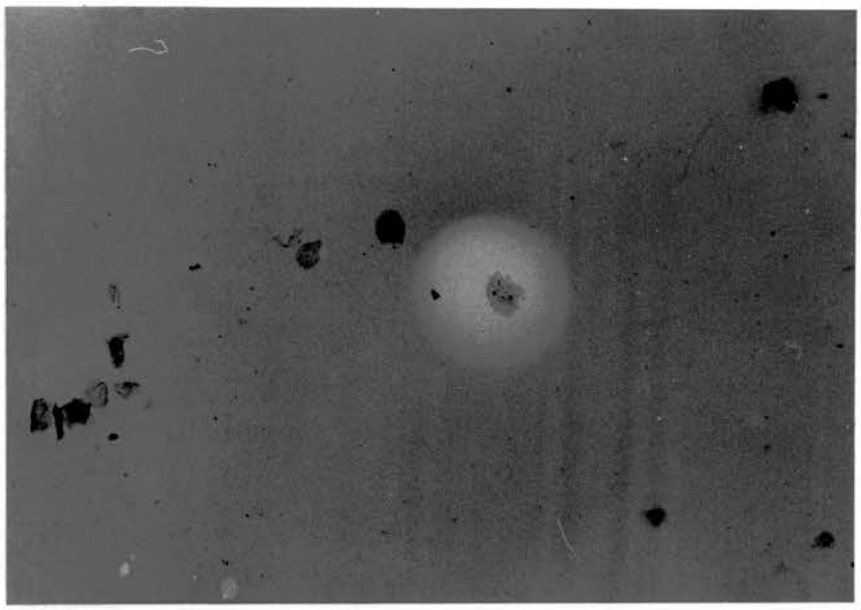
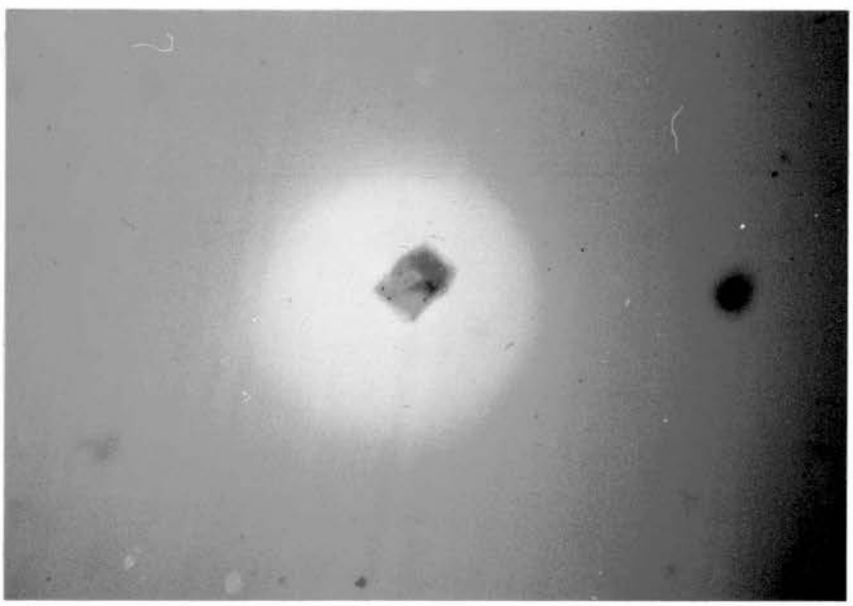


Fig. 15.



Figs. 14 & 15.

Density Gradient Fractions 1 & 2 examined by fibrinolytic autography. Lysis associated with anuclear cell fragments. Human fibrin, incubated overnight at room temperature and 1 hour at 37°C. Mag. Fig. 14 x 120. Fig. 15 x 300.

cubated at 37°C for 19 hours. Smears of each fraction were covered with celloidin and stained with Azure and Gram. Smears of the active fractions were prepared for fibrinolytic autography (Methodology, p. V) In the later experiments (Nos. 11 - 13), the active fractions were diluted in equal volumes of saline, centrifuged for 10 minutes at 4154 g. and the supernatant filtered through an 0.22 micron Millipore filter (see Part III, Experiment 3). 3 x 0.03 ml. drops of the diluted fractions, the supernatant and the filtrate were placed upon SHFP and incubated for 19 hours at 37°C.

Results:

These are recorded in Tables 43 and 44 (Appendix 1).

Lysis was only found in the three least dense fractions (20, 25 and 30%). However most of the pellet was present as a well defined mass below fraction 9 and this was always active. The smears showed an obvious difference between fractions 1 and 9, there being a much more gradual change between each of the individual fractions. Fraction 1 contained a few bacteria, odd nucleated epithelial cells and a number of anuclear epithelial cell fragments (Figs. 10 & 11) Fraction 9 usually contained numerous nucleated epithelial cells and large numbers of bacteria (Figs. 12 & 13). Fibrinolytic autography of fractions 1 and 2 showed a suggestion of lysis around some of the anuclear epithelial cell fragments with occasionally more well defined foci of lysis (Figs. 14 & 15). The supernatants of the diluted fractions after centrifugation showed reduced activity (with one exception) but after filtration none of the active fractions showed activity. The concentrated pellet after centrifugation was always active in the fractions 1 and 2.

Discussion:

This experiment failed to isolate the whole of the plasminogen activator activity of salivary pellet in one particular density fraction. Part of the/

the activity could be separated out in the least dense fractions from the main mass of the pellet, and this activity in the least dense fractions appeared to be related to anuclear epithelial cells or fragments. By examination of smears from fractions 1 and 9, it was obvious that the activity was not directly related to the total number of bacteria present, lysis being present in fractions with relatively few bacteria. The experiment, however, did support the evidence in Part III in showing the absence of a soluble activator in the salivary pellet. If a soluble activator were present, it is likely that it would remain in the least dense fractions and although these were the only fractions found to be active, this activity was reduced with centrifugation and totally eliminated with filtration through an 0.22 micron filter. The large cell portion that passed right through the gradient was also consistently active.

Conclusions:

1. The plasminogen activator activity of the salivary pellet is partly related to anuclear epithelial cells or epithelial cell fragments.

2. No soluble plasminogen activator is present in salivary pellet.

3. The plasminogen activator activity of the salivary pellet is not directly related to the total number of bacteria present.

CHAPTER 3

SALIVARY MUCUS

1. The writer can find no reference in the literature to suggest that salivary mucus has plasminogen activator activity.

2. It has been shown in Part III, Experiment 1 that only occasionally does lysis occur with a mucus-containing sample of submandibular/sublingual saliva upon SHFP and that following either centrifugation or filtration, this slight activity disappears.

3. Fibrinolytic autography. (Human fibrin: Methodology, p. V) Salivary pellet was obtained by centrifuging 10 ml. of MNS for 30 minutes at 4^{o}C at 4154 g. The supernatant was discarded and the pellet frozen with carbon dioxide and sectioned. Five micron thick frozen sections were cut and prepared for fibrinolytic autography (see Methodology, p. V) Prolonged incubation caused the lysis of the whole film but short incubation periods brought about lysis that was clearly focal. This would not have occurred if the mucus had been active for the whole film would have lysed at the same time, the mucus being evenly distributed throughout the pellet. Fig. 16 clearly illustrates foci of lysis and thereby contra-indicates activity in relation to salivary mucus.

4. Fibrinolytic autography of major and minor salivary mucous glands showed lysis only related to blood vessels. Figs. 17 & 18.

Conclusion:

Salivary mucus has no plasminogen activator activity.

Fig. 16.

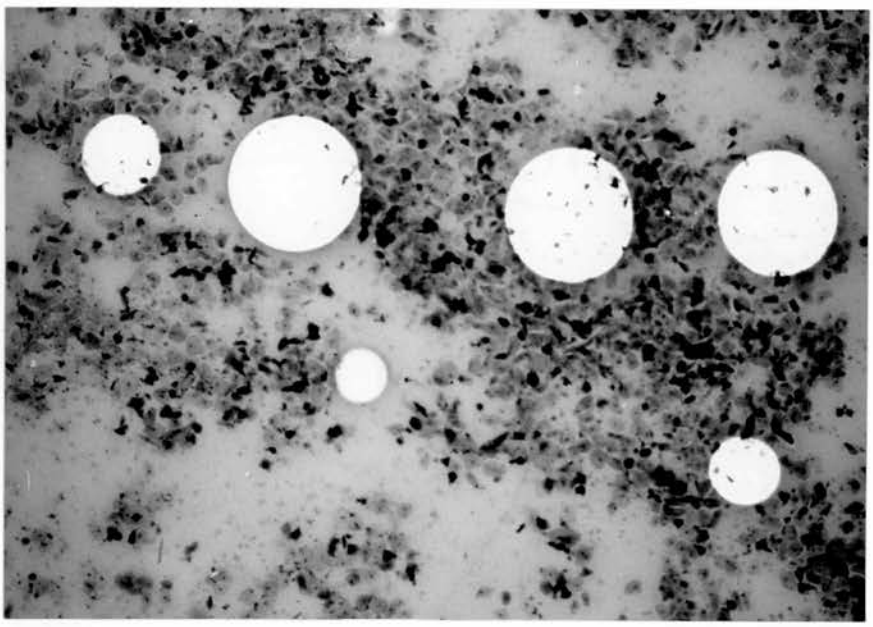


Fig. 16. Fibrinolytic autograph of a smear of salivary pellet showing foci of lysis. Human fibrin , incubated overnight at room temperature and 1 hour at 37°C. Mag. x 300.

Fig. 17.

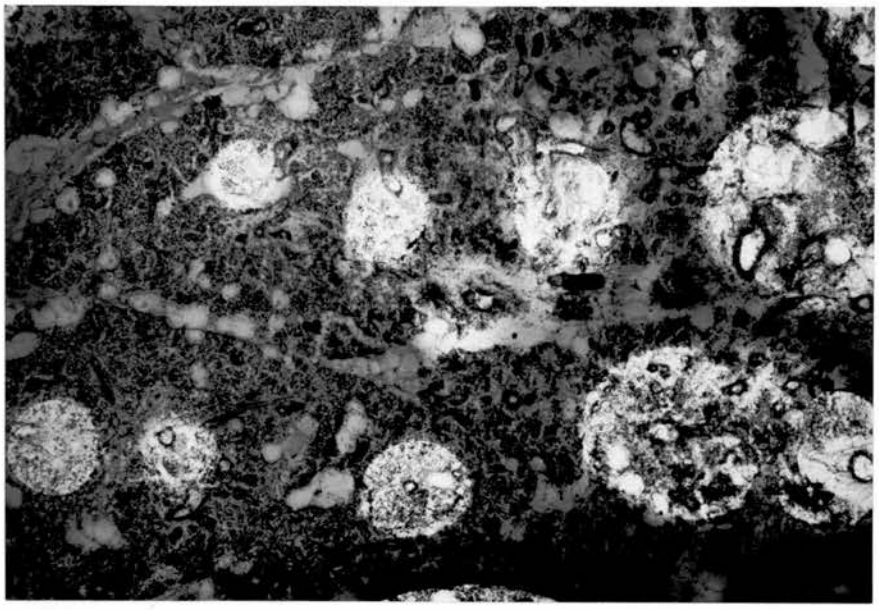


Fig. 17. Fibrinolytic autograph of human submandibular salivary gland showing foci of lysis unrelated to the secretory acini. Human fibrin, incubated 45 mins. at 37°C. Mag. x 30.

Fig. 18.

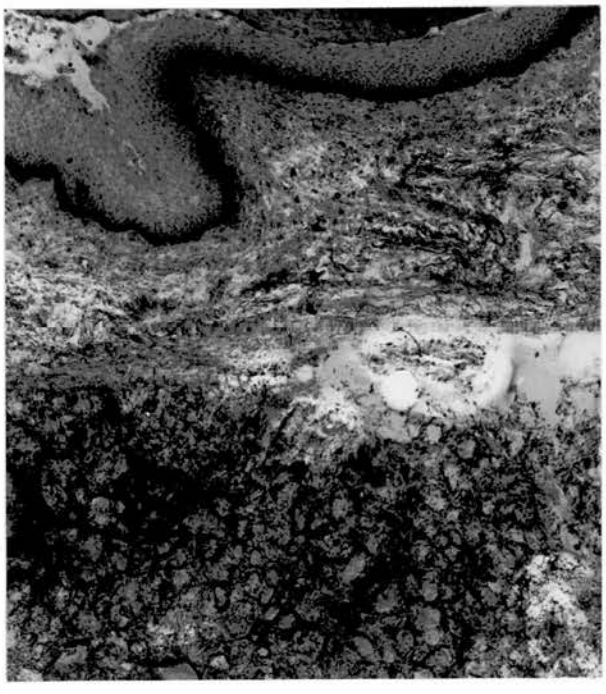


Fig. 18. Fibrinolytic autograph of human floor of mouth mucosa showing a focus of lysis (arrowed) related to a blood vessel. No lysis related to the mucous gland or epithelium. Human fibrin incubated for 30 mins. at 37°C. Mag. x 48.

CHAPTER 4

THE SALIVARY FLORA

1. The salivary flora.

2. Experiment 1. To study the effect of a bacteriostat upon the plasminogen activator activity of MNS.

3. Experiment 2. To test the salivary flora and their filtrates for plasminogen activator activity using :

   a) The Somerville clot lysis technique.
   b) SHFP

4. Experiment 3. To study the plasminogen activator activity of the salivary flora using fibrinolytic autography.

5. Experiment 4. To determine whether or not a quantitative relationship exists between salivary bacteria and the plasminogen activator activity of MNS.

6. Experiment 5. To test MNS for the presence of a streptokinase-like activity.

1. THE SALIVARY FLORA

Morris (1953) states that in excess of 3,200 different strains of micro-organisms have been isolated from saliva while Ross (1971) considers the oral flora of man to be one of the densest and most varied microbial populations in the body.

A wide variety of bacteria is known to possess fibrinolytic activity (Weiss, 1937: Tillett, 1938: Reed et al, 1943: Dixon, 1945: Lewis et al, 1949: Lewis and Ferguson, 1950 and 1951: Cliffton and Cannamela, 1953: Adamis, 1961: Kopper, 1962: and Yaromyuk and Domaradskii, 1963). A census of bacterial categories in saliva carried out by Richardson and Jones (1958) assists in putting the salivary flora in perspective and it is possible to construct from this work and the knowledge of bacteria possessing fibrinolytic activity, a table in which the salivary flora are ranked and fibrinolytic activity, as far as it is known, indicated.

Salivary Flora and Fibrinolytic Activity

Count Category	Bacteria	% Isolation	Fibrinolytic Activity
More than 1 x 10^6 per ml.	Alpha-haem. strep.	100	- ve
	Veillonella	100	- ve
	Micrococci (not aureus)	60 - 90	- ve
	Corynebacteria	78 -100	- ve
	Neisseria (pharyngis and catarrhalis)	T - 84	- ve
	Pneumococci	T - 76	- ve
1×10^2 - 1×10^6 per ml.	Lactobacilli	50-100	- ve
	Strept. faecalis	21 - 42	(- ve)
	Haemophilus/		

Count Category	Bacteria	% Isolation	Fibrinolytic Activity
	Haemophilus influenzae	T - 24	- ve
	Beta-haemolytic strept.	T - 22	+++ ve
	Fusobacteria	G	- ve
	B. melaninogenicus	G	+ ve
	Actinomyces	G	- ve
	Leptotrichia	G	- ve
Less than 1×10^2 per ml.	Staph. aureus	2 - 50	++ ve
	Coliforms	T - 16	- ve
	Candida	T - 30	- ve

Key:

% Isolation: From a survey of the literature these figures, expressed as a percentage, indicate frequency of isolation.

T. Transient.

G. 'Generally present'. Many of the bacteriological reports do not record percentage isolation but use terms such as 'consistently present' or 'generally present'.

Fibrinolytic Activity:

- ve No fibrinolytic activity.

(- ve) Conflicting evidence concerning fibrinolytic activity.

+ ve Fibrinolytic Activity

Comment:

There are three confirmed fibrinolytically active bacteria in saliva.

1. Beta - haemolytic streptococcus is unlikely to contribute to the fibrinolytic activity measured in this work since all persons examined were in good health with no evidence of suffering from acute streptococcal sore throat. Most workers consider this organism not to be a commensal of the mouth and that its presence is only transient. The highest figure in respect of percentage isolation is 22% (Ross, 1971) and when it was isolated, the mean count was in the region of 2×10^5 per ml. On the basis of the work of Richardson and Jones (1958), this would represent about 1/800 of the total cultivable salivary flora.

Fibrinolytic activity was common to all samples of MNS tested upon SHFP in this work, and therefore present in the 78% (minimum) in which beta-haemolytic streptococci were likely to be absent.

2. Staphylococcus aureus. A similar argument pertains to this micro-organism. There is a marked discrepancy between the figures given for percentage isolation, Ross (1971) finding a 2% incidence in a child population to a 50% isolation by Burnett and Scherp (1968). Both papers agree upon a low mean count however, below 1×10^5 per ml.

3. Bacteriodes melaninogenicus. The collagenase producing properties of this organism are better recognised than its fibrinolytic activity and there is a possibility that at least part of its activity is protease rather than plasminogen activating. Burnett and Scherp (1968) consider that it constitutes about 0.4% of the cultivable bacteria from saliva although it may comprise 4.5% of that cultivable from the gingival sulcus. This is consistent with the work of Gordon et al (1965) who suggested that the salivary flora reflected the flora of the tongue rather than the gingiva.

Conclusion/

Conclusion:

The literature provides little evidence to indicate that the fibrinolytic activity of MNS is closely related to the salivary flora.

2. EXPERIMENT 1

Object: To study the effect of a bacteriostat upon the plasminogen activator activity of MNS.

Preliminary Experiment

Object: To determine the minimum concentration of Crystamycin required to inhibit the formation of colony forming units derived from MNS.

Materials:

1. Crystamycin: 300 mg. (500,000 units) Benzylpenicillin (sodium) BP. plus
   500 mg. Streptomycin sulphate BP. (Glaxo Laboratories Ltd.)
2. Freshly prepared bullock heart infusion broth, blood agar and MacConkey plates. (Supplied by the Department of Bacteriology, Edinburgh University).
3. Gas Paks. Disposable gas generator envelopes which produce an atmosphere of 95% hydrogen and 5% carbondioxide (Becton, Dickinson & Co., U.S.A.)
4. McIntosh and Fildes' anaerobic jars, sterile loops and glassware.

Method:

Day 1. From a healthy subject 1.5 ml. of minimally stimulated MNS (Methodology, p.XXV) was collected. 10 ml. of sterile nutrient broth was then innoculated with MNS and incubated at 37°C for 24 hours.

Day 2. a) The incubated broth was placed into five sterile tubes. Crystamycin, 400 mg./ml. was added to 4 of the 5 tubes to/

to establish end concentrations of 0%, 10%, 5%, 2.5% and 1.25%. Each tube was then sealed and incubated for 24 hours at 37°C.

b) The above procedure was repeated except that fresh sterile broth was used and all the tubes except number 1 were innoculated with MNS after Crystamycin had been added and before incubation at 37°C for 24 hours.

Day 3. The contents of the ten tubes were plated upon separate MacConkey and blood agar plates both aerobic and anaerobic and incubated for 24 hours.

Results:

a)	Plates innoculated from tube 1.	Normal mouth commensals.
	Plates innoculated from tubes 2 - 5.	No colonies.
b)	Plates innoculated from tube 1.	Normal mouth commensals.
	Plates innoculated from tubes 2 - 5.	No colonies.

Conclusion:

The lowest concentration of Crystamycin, 1.25% (5 mg./ml.) was sufficient to have a bacteriocidal effect on the salivary flora.

The experiment was repeated with lower concentration of Crystamycin and it was found that a concentration of 0.6 mg./ml. was bacteriocidal and also produced no lysis upon SHFP.

Experiment 1A.

Object: To study the effect of a bacteriostat upon the plasminogen activator activity of MNS.

Materials: /

Materials:

1. MNS. Minimally stimulated (Methodology, p. XXV)
2. Crystamycin. 200 mg./ml.
3. SHFP (Methodology, p.I)
4. Controls. As in preliminary experiment.

Method:

MNS was collected from twenty subjects who were in good health, taking no drugs and who showed no clinical evidence of gingivitis. From each saliva sample 0.997 ml. was removed and placed in separate sterile tubes. To each was added 0.003 ml. of the Crystamin solution achieving a final concentration of 0.6 mg./ml. A further 0.997 ml. of each MNS sample was removed and to each was added 0.003 ml. of tris buffer. Both sets of samples were now vortex stirred and then plated (3 x 0.03 ml.) upon SHFP and incubated for 19 hours at 37°C. Controls were set up as described in Part II, Experiment 3, Fig. 2.

Results:

These are recorded in Table 45 (Appendix 1).

Summary of Results:

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of MNS, with and without Crystamycin, upon SHFP after 19 hours incubation at 37°C.

Subjects (20)	MNS with Crystamycin	MNS without Crystamycin
Mean	191	188
S.D.	35	36

Conclusion:/

Conclusion:

There is no significant difference between the lysis produced by MNS with Crystamycin and that produced by MNS alone.

Experiment 1B.

Object: To study the effect of incorporation of a bacteriostat within an SHFP upon the amount of lysis produced by MNS.

Materials:

1. MNS. Collection as in Experiment 1A from 20 subjects.
2. Crystamycin, SHFP and controls as in Experiment 1A.
3. Human fibrin plates incorporating Crystamycin at an end concentration of 0.6 mg./ml.

Method:

MNS, about 1 ml., was collected by minimal stimulation from each subject and after vortex stirring, was plated (3 x 0.03 ml.) upon SHFP and plates containing Crystamycin. Controls were set up as in Experiment 1A. All the plates were incubated for 19 hours at 37^{o}C.

Results:

These are recorded in Table 46 (Appendix 1).

Summary of Results:

Average areas of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of MNS upon SHFP and upon human fibrin plates containing Crystamycin after 19 hours incubation at 37^{o}C.

Subjects/

Subjects (20)	SHFP	Fibrin Plate with Crystamycin
Mean	192	196
S.D.	35	37

Conclusion:

There is no significant difference between the areas of lysis produced by MNS on the SHFP and those of the fibrin plates incorporating Crystamycin.

Experiment 1C.

After Experiments 1A and B had been completed, the stability experiments described in Part II were performed. The results are discussed in Part II.

Discussion and Conclusions:

It was established that under conditions conducive to the growth of salivary bacteria (i. e. nutrient broth), a concentration of Crystamycin as low as 0. 6 mg. /ml. was sufficient to prevent the formation of any colonies on blood agar plates on sub-culture. Incorporation of Crystamycin into the MNS could have had one of three effects.

1. Bacterial cell death with disintegration of the dead bacteria, release of endogenous proteolytic enzymes and thus increased fibrinolytic activity.
2. Bacterial cell death with the prevention of the release of fibrinolytic enzymes and therefore no release of these enzymes whilst incubating upon the fibrin plates resulting in a reduced plasminogen activator activity.
3. Possible release of plasminogen activator and/or inhibitor from the oral flora.

In/

In the event none of these possibilities appears to have taken place as the difference in lysis between the system incorporating Crystamycin and the one which does not is insignificant, within the sensitivity of the technique. The activator activity of MNS measured in vitro is not significantly altered by killing the bacteria and storing MNS at a temperature conducive to bacterial growth results in a decrease of activity and not an increase.

These findings suggest that the oral flora plays a minor role, if any, in the plasminogen activator activity of MNS.

3. Experiment 2(i)

Object: To test the salivary flora and their filtrates for plasminogen activator activity using :-

A. The Somerville clot lysis technique.
B. SHFP.

Somerville clot lysis technique (1972). The basis of the test is the incorporation of bacteria or bacterial filtrate into a fibrin clot and measurement of the clot lysis time. A reduced clot lysis time relative to controls indicates some fibrinolytic activity in relation to the incorporated bacteria.

Materials:

1. MNS. MNS was collected by stimulated flow (Methodology, p. XXVI) from a subject who had no clinical evidence of gingivitis.
2. Blood agar plates and nutrient broth. Freshly prepared and supplied by the Department of Bacteriology, Edinburgh University.
3. SHFP and the reagents required for their preparation, as these were used for the preparation of the Somerville tubes.

Isolation. 0.5 ml. of MNS was placed in 5 ml. of fresh nutrient broth. The tube was rolled after which 0.5 ml. was placed in a further 5 ml. of broth and the process continued to dilution number 6. Dilutions 4, 5 and 6 were selected and three blood agar plates were swept with 0.02 ml. of the innoculated broth at these dilutions. The plates were incubated aerobically for 24 hours at 37^{o}C. At 24 hours, dilution 5 with approximately 50 colony forming units per plate facilitated isolation of the colonies for subculture.

The/

The following bacteria were isolated and identified by their colony size, shape, colour, texture and ability to lyse blood agar plates and also by their Gram staining properties and microscopic appearance. Alpha streptococcus, Micrococcus, Diphtheroid bacilli, Staphylococci (citreus), Diplococci, Neisseria, Streptococci (faecalis) and Gram positive branching filamentous bacteria considered by a bacteriologist to be probably Lactobacillus.

The isolates were plated upon fresh blood agar plates and into nutrient broth and incubated aerobically at 37°C for 24 hours as was a broth culture of Beta haemolytic streptococci (BHS). A further MNS sample was diluted and plated as above at dilutions 4, 5 and 6 upon blood agar. One ml. of MNS was placed in 9 ml. of broth and incubated for 24 hours at 37°C. Therefore, 48 hours from the commencement of the experiment, there were four sets of preparations for testing, namely :-

1. A set of isolates cultured aerobically on blood agar and in nutrient broth.
2. A subculture of BHS on blood agar and in nutrient broth.
3. Dilutions of MNS swept over blood agar to give a mixed aerobic culture.
4. MNS cultured in broth.

Fibrin Plates and Somerville Tubes

1. SHFP. (Methodology, p. I).
2. SHFP containing 0.1 ml. of molar eACA.
3. Somerville tubes. To an 0.1% human fibrinogen solution in tris buffer pH 7.8, 0.15M. was added 0.2 ml. per 10 ml. of 0.1M. calcium chloride. The resulting solution was dispensed in 1 ml. aliquots into 5 ml. test tubes and into half of the tubes was placed 0.01 ml. of molar eACA.

Method/

Method:

1. Isolates

a) SHFP. Each isolate was suspended in nutrient broth after which the suspension was plated (3 x 0.03 ml.) upon SHFP with and without eACA.

b) Somerville Tubes. 0.03 ml. of each isolate suspension prepared above was placed in each of six tubes, 3 with and 3 without eACA.

c) SHFP. Each nutrient broth in which a single isolate had been cultured was filtered through an 0.22 micron Millipore filter and the filtrate plated (3 x 0.03 ml.) upon SHFP with and without eACA.

d) Somerville tubes. Using the filtrates prepared above, 0.03 ml. was placed into each of six tubes, 3 with and 3 without eACA.

Isolate Controls

1. Sterile nutrient broth. 3 x 0.03 ml. drops upon SHFP with and without eACA.

2. Sterile nutrient broth. 0.03 ml. into each of three Somerville Tubes with and without eACA.

3. Tris buffer, pH 7.8, 0.15M. Negative control. 0.03 ml. drop per plate, and 0.03 ml. into each of three Somerville Tubes with and without eACA.

4. Urokinase. 5 Ploug units per ml. Dispensed as for tris buffer. Positive control.

2. BHS on blood agar and in nutrient broth.

BHS was treated as one of the isolates. The same controls were used.

3./

3. MNS. Mixed aerobic culture.

A sterile loop was taken through the various colonies on the plate as a "sweep". This was repeated several times until a dense suspension of bacteria was obtained in 0.5 ml. of nutrient broth. Thereafter the "sweep" was treated in the same manner as the isolate suspensions.

4. MNS cultured in broth.

The mixed flora growing in this broth would not be present in the same proportions as those found in MNS, nevertheless they would be of MNS origin and the filtrate formed may have contained plasminogen activators.

One ml. of MNS was placed in 9 mls. of nutrient broth and therefore epithelial cells were also present. After 24 hours culture, the inoculated broth was vortex stirred and filtered through 14 micron Millipore filters which would allow the passage of bacteria but not epithelial cells. This filtrate was then centrifuged at 4154 g. for 30 minutes after which some 7 mls. of supernatant were removed and filtered through 0.22 micron Millipore filters to provide a filtrate containing no bacteria but containing any fibrinolytic enzymes that might have been released. The remaining 3 mls. of broth containing the pellet of bacteria was vortex stirred and labelled "bacterial suspension".

This "suspension" and the final filtrate were now treated in the same manner as the isolates.

All the SHFP, with and without eACA, were incubated at 37^{o}C for 19 hours.

All the Somerville Tubes had added to them 0.02 ml. of 50 I.U./ml. thrombin, were vortex stirred and allowed to clot. These tubes were/

were now incubated at 37°C and examined at 19, 24 and 40 hours. The results are recorded in Tables 47 and 48 (Appendix 1).

Summary of Results:

SHFP.

The isolates, bacterial sweeps and bacterial suspensions caused no lysis in suspension nor did their filtrates. BHS in suspension caused an average lysis of 130mm^2. and its filtrate an average area of 110mm^2. Amongst the controls, only urokinase produced lysis.

SHFP with eACA.

No lysis on any plate after 19 hours incubation at 37°C.

Somerville Tubes.

Key:		
	0	No Lysis.
	Lysis +	Most of the clot remained but was floating freely in the tube.
	Lysis ++	Most of the clot was lysed but some clot was still present.
	Lysed.	Completely fluid. No clot was visible at all.

SHF Tubes.

	After 19	24	40 hours at 37°C
1. All isolates suspension and filtrates	0	0	Lysis ++
2. BHS. Suspension and filtrates.	Lysed		
3. Bacterial sweep. Suspension and filtrate.	0	0	Lysis ++

4./

4.	MNS. Bacterial suspension and filtrate.	0	0	Lysis ++
5.	Controls. Nutrient broth.	0	0	Lysis ++
	Tris buffer.	0	0	Lysis +
	Urokinase.	Lysed		

SHF. plus eACA.

1.	Isolates. As above.			
2.	BHS. Suspension.	0	Lysis ++	Lysed.
	Filtrate.	0	Lysis ++	Lysed.
3.	Bacterial sweep. As above.			
4.	MNS. As above.			
5.	Nutrient broth and tris buffer. As above.			
	Urokinase.	0	Lysis +	Lysed.

Discussion:

The SHFP appeared insensitive to any plasminogen activator activity that might have been produced by the bacteria. Saliva is a poor culture medium since it contains many antimicrobial components such as Lysozyme, the antilactobacillus thiocyanate-dependent factors, Green's factor, lactoferrin, salivary immunoglobulins and fluoride (MacFarlane and Mason, 1972). Nutrient broth was used therefore in this experiment in order not to depress any bacterial fibrinolytic activity.

The BHS suspension and filtrate both brought about measurable lysis of the SHFP but failed to produce any lysis on the eACA plates, thus demonstrating specific plasminogen activator activity.

With the exception of BHS suspension and filtrate, no lysis occurred with Somerville tubes until after 24 hours. The controls suggested no difference between nutrient broth and any of the bacterial suspensions/

pensions prepared in nutrient broth. The tris negative control however was considerably less lysed at 40 hours than was the nutrient broth control. The urokinase control was lysed at 19 hours. In the tubes with eACA added, the results were the same as those for tubes without eACA with the exception of the BHS and urokinase positive control. With both these tests, no lysis occurred at 19 hours but by 24 hours, lysis was evident and was complete at 40 hours. The experiment was repeated three times using different salivas albeit the same isolates were cultured. Before drawing any conclusions, a further series of experiments was prepared.

Experiment 2 (ii)

The last experiment raised the possibility that the nutrient broth at least in the Somerville Tube technique might have caused some lysis. The tris negative control was undoubtedly firmer. The bacteria had been examined in broth but their normal environment is saliva. It was decided to repeat all the experiments with the suspensions in tris buffer and the supernatant of MNS generated at high speed centrifugation (35,664 g.)

The preparation of all the test solutions was as before except that the final resuspension was in either the supernatant of MNS or tris buffer. MNS supernatant alone was added to the controls. Filtrates of bacterial cultures were, of course, in broth.

Summary of Results:

1. SHFP Isolates.

In suspension in tris. No lysis.
In suspension in MNS supernatant. No lysis.
Filtrates. No lysis.

2. BHS.

In/

In suspension in tris. Lysis: average area 121 mm^2.
In suspension in MNS. Lysis: average area 169 mm^2.
Filtrates. Lysis: average area 110 mm^2.

3. Bacterial sweep.

In suspension in tris (very dense concentration). Lysis: average area 128 mm^2.
In suspension in MNS supernatant. Probable lysis.
Filtrates. No lysis.

4. MNS Bacterial suspension.

In tris. No lysis.
In MNS supernatant.
Filtrates. No lysis.

5. Controls. Nutrient broth, tris buffer, MNS supernatant. All no lysis.
Urokinase. Average area of lysis 225 mm^2.

SHFP with eACA.

1. Bacterial sweep. Suspension in tris (very dense concentration). Lysis: average area 110 mm^2.
2. Bacterial sweep in suspension in MNS supernatant. Probable lysis.

When the experiment was repeated (twice), neither of these results was positive. This may be due to the fact that on the first occasion recorded above, an extremely dense bacterial suspension was obtained. (When the plate was read, there was a thin white mat of cells in the lysed area). The activity appears to have been non-specific however, as it occurred also on the eACA plate. An alternative possibility is that in making the sweep, some blood agar was inadvertently picked up by the platinum loop for Somerville(1972) indicates that the "minimal medium (agar plates) produced lysis within 20 hours".

Somerville Tubes.

All the results were identical to the first series in Experiment 2(i) both with SHF and SHF plus eACA.

Discussion:

Bacteria, derived from MNS, were cultured and applied to the test system which so readily demonstrated plasminogen activator activity in MNS. No activity was detected relative to the negative controls. That the system will detect such activity has been demonstrated by the result obtained from testing BHS. However, bacterial produced plasminogen activator cannot be said to be absent in MNS since it is well established (Afonsky, 1961: Soder, 1972) that some of the protease activity of MNS and dental plaque is almost certainly of bacterial origin. That no such activity was detected is indicative, therefore, of a lack of sensitivity in this system.

Conclusions:

The experiment provides no evidence to show that the plasminogen activator activity of MNS is directly attributable to the oral flora. However, some contribution from the oral flora cannot be eliminated since the technique proved insensitive to the presence of small quantities of protease that might be expected to be present.

Given that the test system used throughout for measuring the fibrinolytic activity of MNS has been the SHFP, then within the limitations of this system the oral bacteria make no significant contribution to the plasminogen activator activity.

Experiment 2 (iii)

The experiments were repeated using sweeps from anaerobic plates and filtrates from anaerobic cultures.

The/

The results were negative.

The conclusion is as stated above.

4. EXPERIMENT 3

Object: To study the plasminogen activator activity of the salivary flora using fibrinolytic autography.

Method:

Fibrinolytic autography (Methodology, p. V).

Both human and bovine fibrinogen was used. Smears of the bacteria grown on blood agar plates in Experiment 2 were covered with fibrin and suspensions of the same bacteria were included within the fibrin films. Bacteria were also present on many of the fibrin autographs of epithelial cells from salivary pellet and dorsal tongue sections and smears described in Part IV, Chapter 6.

Results:

Foci of lysis developed in human fibrin around clumps of BHS but not around individual bacteria (Fig. 19). This lysis was blocked if eACA was incorporated in the fibrin film and was not seen on bovine fibrin film. Lysis was never obtained in association with any of the other bacteria investigated even after the longest incubation times (Fig. 20) Although lysis was seen to be related to epithelial cells in the dorsal tongue smears covered with human fibrin, it was never related to bacteria even when clumps of bacteria of a similar size to the clumps of BHS which produced lysis were present on the films (Fig. 21)

Sections of dorsal tongue showed no lysis related to clumps of bacteria on the surface, although lysis over the connective tissue was present on the same sections when covered with either human or bovine fibrin (Fig12). Frozen sections of salivary pellet showed foci of lysis, in the centre of which could be seen epithelial cells (Fig.23), while no foci of lysis related to bacteria were seen.

Discussion and Conclusions:

Fig. 19.

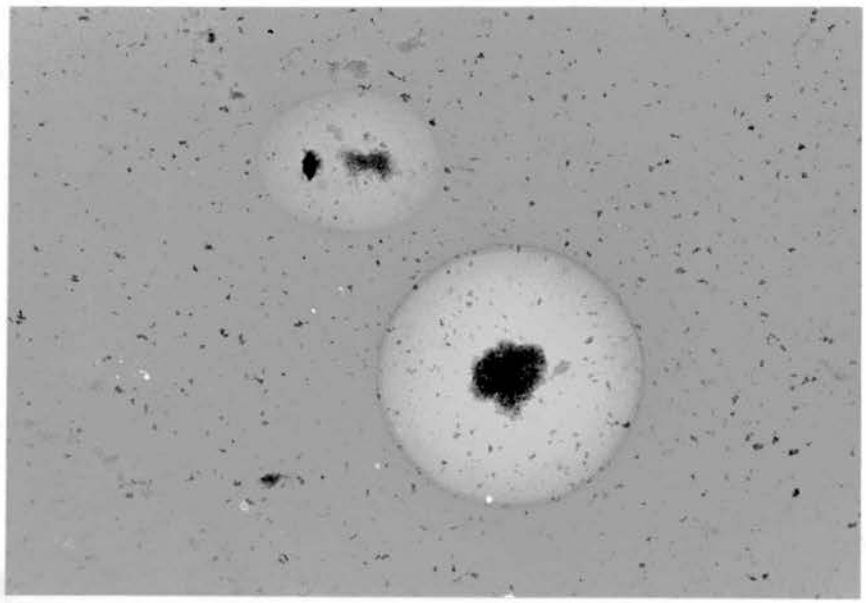


Fig. 19. Fibrinolytic autograph of Beta Haemolytic Streptococci showing lysis around clumps but not individual bacteria. Human fibrin , incubated overnight at room temperature. Mag. x 120.

Fig. 20.

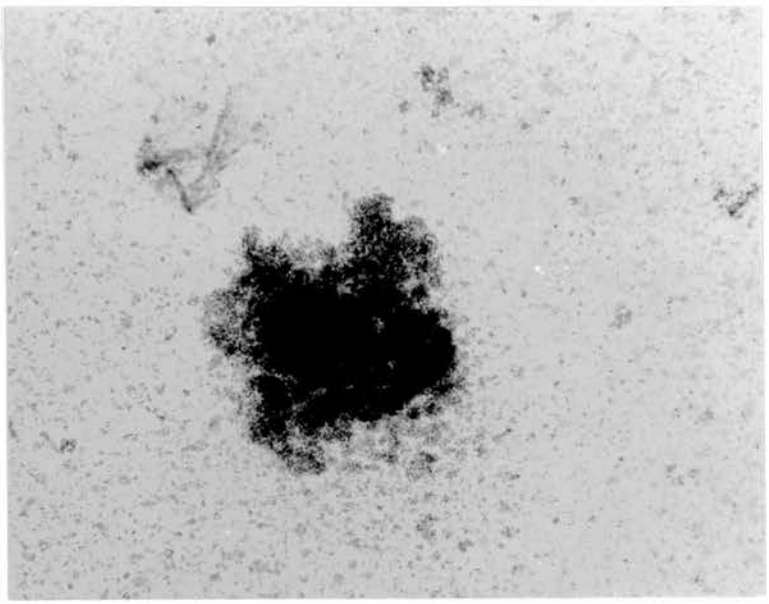


Fig. 20. Fibrinolytic autograph of Alpha Haemolytic Streptococci isolated from saliva shows no lysis , even when in large clumps. Human fibrin , incubated overnight at room temperature. Mag. x 300.

Fig. 21.

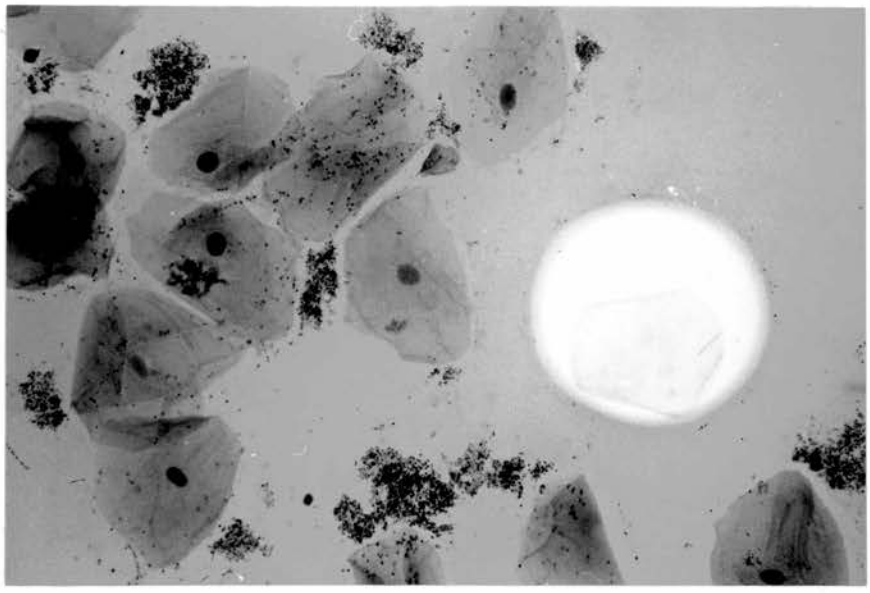


Fig. 22.

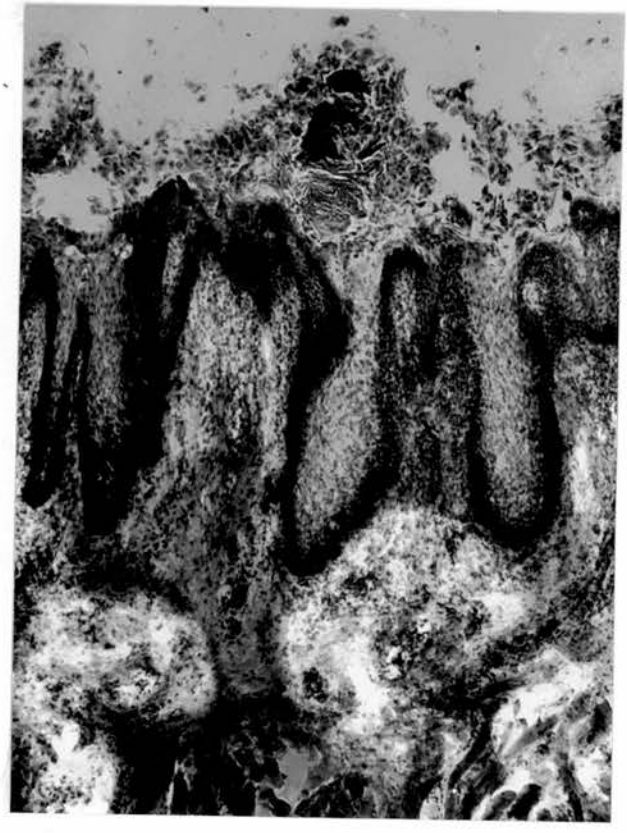


Fig. 21. Fibrinolytic autograph of dorsal tongue smear shows lysis around an anuclear epithelial cell but no lysis around clumps of bacteria. Human fibrin , incubated overnight at room temperature and 1 hour at 37°C. Mag. x 300.

Fig. 22. Fibrinolytic autograph of human dorsal tongue showing lysis around vessels in the connective tissue , but no lysis around the clumps of bacteria on the surface. Human fibrin , incubated for 18 hours at 4°C. Mag. x 36.

Fig. 23.

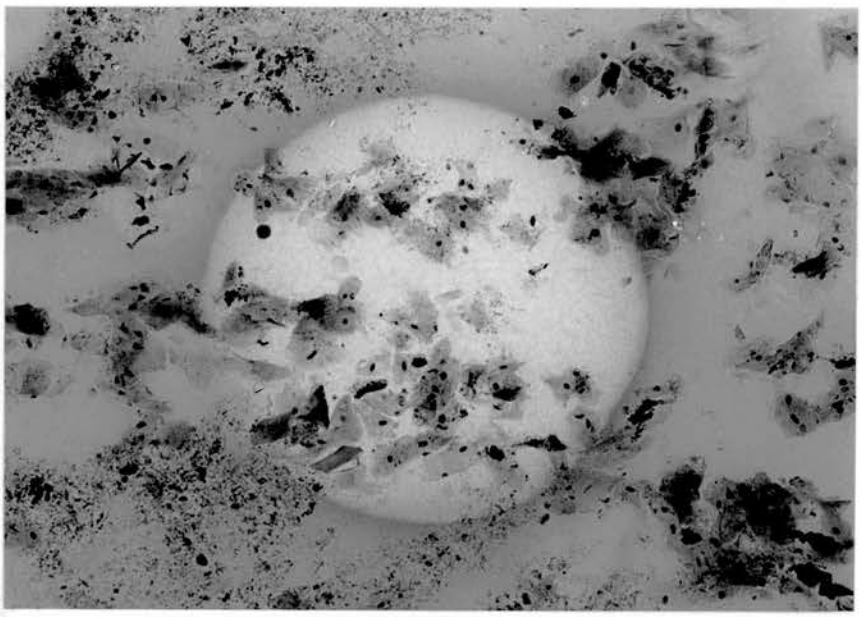


Fig. 23. Fibrinolytic autograph of a frozen section of salivary pellet showing a focus of lysis related to an epithelial cell but not to the numerous bacteria present. Human fibrin , incubated 24 hours at $4^{o}C$ and 1 hour at $37^{o}C$. Mag. x 120.

Discussion and Conclusions:

These results support the findings in Experiments 1 and 2 that the oral flora are not the source of the plasminogen activator activity of MNS. The fibrinolytic autograph technique is sufficiently sensitive to detect the fibrinolytic activity of clumps of BHS and isolated epithelial cells. If the normal oral flora are a significant factor in the plasminogen activator activity of MNS, it is likely that some of the bacteria (apart from BHS) would have shown evidence of lysis in these experiments. The BHS produced lysis with human fibrin but not with the bovine fibrin, thus indicating that the lysis of human films was probably mainly due to streptokinase production rather than plasminogen activator.

5. EXPERIMENT 4

Object: To determine whether or not a quantitative relationship exists between salivary bacteria and the plasminogen activator activity of MNS.

Comment:

Schiott et al, (1970) examined the extent to which the oral flora was influenced by repeated chlorhexidene rinses. Subjects rinsed twice daily with 10 mls. of an 0.2% solution of chlorhexidene gluconate. The number of bacteria per ml. of saliva was reduced by 85% after 24 hours reaching a 95% reduction on day five. An 85 - 95% reduction was maintained throughout the trial period of 22 days.

Materials:

1. Subjects: Ten subjects, (5 Male, 5 Female) aged 22 - 41, all of whom were in good health, were taking no drugs and who had no clinical evidence of gingivitis.
2. MNS. Produced by stimulated flow (Methodology, p. XXVI)
3. SHFP. (Methodology, p. I)
4. Blood agar plates. Freshly prepared and supplied by the Department of Bacteriology, Edinburgh University.
5. Nutrient Broth Transport Medium. Supplied as above.
6. McIntosh and Fildes' anaerobic jars.
7. Gas Paks.

Method:

For four days prior to the first saliva collection, all the subjects maintained their normal oral hygiene procedures and in addition rinsed their mouths three times a day with 10 mls. of water. On the fifth and sixth day, whilst still rinsing with water, 4 - 5 mls. of MNS was collected by stimulated flow at 09.00 and 14.00 hours. The/

The specimens were vortex stirred, after which 1 ml. was removed and transferred to a sterile bottle containing 9 ml. of nutrient broth. Logarithmic serial dilution was continued until seven dilutions had been prepared. From each of the last four dilutions, 4, 5, 6 and 7 were removed five drops (0.02 ml.) of broth which were plated upon blood agar. The plates were prepared in duplicate to provide for anaerobic and aerobic culturing and incubated at 37°C for 24 hours.

The saliva samples from which the dilutions had been prepared were stored at -40°C.

After 24 hours incubation, the agar plates were removed from the incubators and read.

The appearance of such a plate is illustrated in Fig. 24.

Reading the plate.

At each dilution there were five sets of colony forming units. Every colony in each set was counted. The total count for each set was recorded and then the five totals were added together and divided by five to give the average colony count for that dilution.

For each saliva sample, there was obtained an average colony count at dilutions 4, 5, 6 and 7.

At the end of day six there had been obtained from each subject, four samples of MNS, most of which had been deep frozen for testing at a later date in respect of plasminogen activator activity and from which also had been made serial dilutions for bacterial culture and subsequent counting. The bacteriological investigations were commenced as soon as the saliva was collected.

On day seven and for the next ten days, each subject rinsed his mouth with 10 mls. of 0.2% chlorhexidene gluconate in place of water. On the ninth and tenth days of this regime, samples of saliva were collected and treated in exactly the same manner described above for/

Fig. 24.

Fig. 24.

Aerobic blood agar plate with five drops (0.02 ml.) of broth at dilutions 4 (top) and 5 (bottom) showing isolated colonies of bacteria after 24 hours incubation at 37°C.

for when the subject was on mouth rinses of water. Thus four sets of saliva per subject were collected for immediate bacteriological investigation and later plasminogen activator assessment.

Following the ten days of chlorhexidene washes, there was a period of seven days on the original regime using water. At the end of this period, on the last two days, saliva samples were again collected.

For every subject there should have been twelve sets of saliva deep frozen for later fibrinolytic investigation and twelve sets of bacteriological data.

In the event six complete sets of twelve were obtained. Two people became unwell with colds during the trial period and dropped out. One so disliked the taste that he refused to continue and one subject, having completed the chlorhexidene rinses, did not return for the final part, the return to water rinses. Nevertheless, for every specimen of saliva (98) collected, there was also a bacteria count.

Preliminary trials had been conducted within the Laboratory to establish the degree of dilution that would be required in order to obtain accurate counting and all specimens had been kept and the bacteriological data recorded. It was, therefore, possible to add a further 66 readings to the 98 obtained on the trial and thereby obtain 164 results from which to establish whether or not a quantitative relationship existed between bacteria per ml. of MNS and the plasminogen activator activity of MNS.

Plasminogen activator activity was assessed upon SHFP. Freshly thawed and well stirred saliva was plated (3 x 0.03 ml.) for each specimen. All the samples were tested at the same time on the same batch of fibrin plates. All were incubated for 19 hours at $37^{o}C$. Controls of urokinase and tris buffer were set up as described in Part II, Experiment 3, Fig. 3.

Results: /

Results:

These are recorded in Tables 49 - 52 (Appendix 1).

Calculation of Results:

1. The correlation coefficient, r, may be calculated from the following formula.

$$r_{xy} = \frac{\text{Sum } (X - \overline{X})(Y - \overline{Y})}{\sqrt{\text{Sum } (X - \overline{X})^2 \text{ Sum } (Y - \overline{Y})^2}}$$

r = mean of all the values of { (observation of X minus mean of the observations of X) ÷ standard deviation of X x (corresponding observation of Y minus mean of the observations of Y) ÷ standard deviation of Y }

Let X = average area lysed by MNS.
Let Y = average count of bacteria, at a defined dilution, contained in sample of MNS producing the lysis, X.

2. Standard error $1/\sqrt{n - 1}$ where n is the number of individuals in the sample.

Summary of Results:

Evidence was sought for an association between :-

a) Activator activity of MNS and the number of aerobes present.
b) Activator activity of MNS and the number of anaerobes present.
c) Activator activity of MNS and the total number of bacteria, aerobes plus anaerobes.

a) $r_{xy} = 0.32$. Standard error 0.08. Therefore r = 4x standard error.

b) $r_{xy} = 0.26$. Standard error 0.08. Therefore r = 3.3x standard error./

error.

c) r_{xy} = 0.29. Standard error. 0.08. Therefore r = 3.6x standard error.

The three results indicate a low but significant correlation between the amount of lysis produced by a sample of MNS and the number of colony forming units grown from it (Hill, 1961).

Discussion:

The ratio of cultivable organisms in saliva to countable is about 1:4 (Socransky et al, 1963) and this ratio does not allow for numbers of dead organisms which may be present. In absolute numbers, therefore, a rise in the number of colonies counted on a plate signifies a very much greater increase in the total number of bacteria. A very large increase in the number of bacteria then may be necessary to bring about an increase in fibrinolytic activity. MacPheeand Cowley (1969) state that the quantitative change in the oral flora which is associated with gingivitis and periodontitis is such that the concentration of organisms in deposits on the teeth is of the order of 2×10^{11} organisms per gramme weight of plaque: approximately the same concentration of organisms as a pure culture of streptococci. They further state that there is an increase in the bacterial enzymes of saliva as a result of this change. This statement is not inconsistent with that of Gordon et al (1965) in which they state the flora of saliva reflects the microbiota of the tongue rather than the gingival crevice for it is the enzymes in the saliva, not necessarily the organisms themselves to which MacPhee and Cowley refer. The chlorhexidene mouthwashes used here to depress the salivary flora reduce the oral flora non-selectively (Loe and Schiott, 1970) to bring about the inhibition of plaque and so prevent gingivitis. Although none of the subjects in this experiment had clinical evidence of gingivitis (as defined in Part II) plaque would be present and hence the enzymes produced by it. Thus/

Thus, while reducing the oral flora and hence the viable flora in saliva, the plaque flora and therefore the enzymes released from it would also be reduced.

Amongst the enzymes produced by plaque and present consequently in saliva are proteinases, peptidase, urease and collagenase.

Although the SHFP plus eACA cannot detect non-specific proteolysis in MNS, this may be because the technique is not sufficiently sensitive to show such activity. Any non-specific proteolytic activity in MNS may enhance the plasminogen activator activity while not being sufficiently powerful to cause lysis on its own in the fibrin plate technique.

In addition, of course, the presence of plaque and some gingivitis necessarily means the presence of some inflammatory exudate with the ingress of leukocytes and plasminogen into saliva.

Another consideration is the action of bacterial endotoxins upon other cellular particles. Wunschmann-Henderson et al (1972) discuss the effect of bacterial toxins upon human leukocytes and how the release of small amounts of plasminogen activator is enhanced by the presence of bacterial toxins. Leukocytes are present in the saliva of all subjects and in greater numbers where gingivitis is present. A complex inter-relationship between bacteria - gingivitis - gingival fluid - leukocytes - activator release may operate. The salivary flora are also found firmly bound to epithelial cells, and might be instrumental in producing an epithelial cell associated activity.

Against all this must be laid the evidence of the bacteriostat experiments, the SHFP, Somerville tubes, and the fibrinolytic autographs. On balance, it appears that although an association between quantities of bacteria and fibrinolytic activity exists, this is not evidence of causation. The reduction of gingivitis, the overall reduction of bacterial enzymes eminating from plaque, the unknown effect of bacterial endotoxins upon other cells present, all obscure the issue. What is clear is that a massive reduction in salivary flora does not prohibit fibrinolysis, no direct/

direct causal relationship exists and this, taken in context with the previous experiments, strongly suggests that while not stating that the oral flora produce no plasminogen activator, their contribution of activator to the MNS activator activity can only be very minor.

Conclusion:

1. A low but significant correlation exists between the amount of lysis produced by a sample of MNS and the number of bacteria colony forming units grown from it.
2. While it has not been demonstrated that the oral flora produce no plasminogen activator, any contribution they do make to MNS activator activity can only be very minor.

6. EXPERIMENT 5

Object: To test MNS for the presence of a streptokinase-like activity.

Materials:

1. Reagents normally used to prepare standard bovine fibrin plates (Methodology, p. IV)
2. Human plasminogen (Lyophilized. 25 caseinolytic units, 10 - 15 cu./mg. protein A.B. Kabi, Sweden).
3. Streptokinase 2,500 units/ml. in tris buffer, pH 7.8, 0.15M.
4. Urokinase. 5 Ploug units/ml. pH 7.8, 0.15M.
5. Tris buffer. pH 7.8, 0.15M.
6. MNS. MNS was collected by stimulated flow (Methodology, p. XXVI) from 15 persons, 7 female and 8 male, who were in good health, taking no drugs and who had no clinical evidence of gingivitis.

Method:

Five sets of bovine fibrin plates were prepared. They were prepared as for SBFP except that four also contained human plasminogen with end concentrations of 0.1, 0.05, 0.025 and 0.012 cu./ml. respectively. The fifth plate was an SBFP and functioned as a control.

Upon each of these five plates were plated 3 x 0.03 ml. of MNS and the control solutions of urokinase and tris buffer (Part II, Experiment 3, Fig. 3). The plates were incubated for 19 hours at 37°C. Streptokinase was similarly applied to the same types of bovine plate at a concentration of 2,500 units/ml. and serially diluted to 0.075 units/ml. on bovine plates containing human plasminogen 0.1 cu./ml.

Results:

These are recorded in Tables 53 and 54 (Appendix 1).

Summary/

Summary of Results:

MNS - no lysis produced.

Streptokinase - at a concentration as low as 0.075 units/ml. lysis of 100 sq. mm. was readily recorded.

Discussion:

MNS appears to have no streptokinase - like activity within the sensitivity of the system described. The apparent absence then of an enzyme which is of bacterial origin and in an area rich in streptococci is further evidence of the very small role the oral flora must play in the fibrinolytic activity of human mixed native saliva.

The result of this experiment is extremely important since the behaviour of MNS upon fibrin plates in one respect is identical to that of streptokinase. That is that both MNS and streptokinase lyse human fibrin plates and do not lyse bovine fibrin plates. It was, therefore, fundamental to demonstrate that the apparent plasminogen activating activity of MNS was not, in fact, simply due to the presence of streptokinase.

CHAPTER 5

SALIVARY LEUKOCYTES

1. The salivary leukocytes.

2. Experiment 1.

   To study the plasminogen activator activity of leukocytes suspended in saline and MNS supernatant and the effect of freeze-thawing on this activity.

3. Experiment 2.

   To compare the plasminogen activator activity in MNS of a dentate population with that of an edentate population.

4. Discussion and conclusions.

1. SALIVARY LEUKOCYTES

The oral micro-organisms contribute only 16 - 34% of the total metabolic activity of MNS (Eichel and Lisanti, 1964). The remainder appears to be accounted for largely by the oral leukocytes (Hoffman, 1966). The gingival sulcus is the major site of entrance of leukocytes into the oral cavity (Sharry and Krasse, 1960: Wright, 1964: Schroder, 1970: Frank and Cimasoni, 1970 and Lange and Schroder, 1971). While the edentulous mouth is almost devoid of leukocytes (Calonius, 1958: Wright, 1962: Klein, 1962: Lantzman and Michman, 1970), Klinkhamer (1968) has shown a close correlation between the number of teeth present and the number of salivary leukocytes. In the healthy edentulous mouth, 1,000 - 143,000 leukocytes/ml.; in the healthy dentulous mouth, 110,000 - 1,364,000 leukocytes/ml.; and in the mouth where gingival inflammation is present 770,000 - 11,896,000 leukocytes/ml. (Calonius, 1958). The upper limit found by Calonius occurred in a single case presenting with a "suppurating fistula" and the second highest count he records is 5,912,000 leukocytes/ml. Calonius examined only thirteen chronically inflamed mouths and if the count of 11,896,000 is excluded, the mean of the remaining twelve is 2,389,000 leukocytes/ml. This figure conveys a more accurate indication of the number of salivary leukocytes in inflamed mouths.

In the gingival tissue and gingival fluid most leukocytes have the appearance of intact cells resembling those in the blood (Lange, 1967: Gavin, 1968: Frank and Cimasoni, 1970: Attstrom, 1970: Lange and Schroder, 1971). In saliva, however, neutrophil polymorphonuclear (PMN) leukocytes appear as cells, and Raeste (1972) has demonstrated that a mononuclear degenerated cell form is predominant. Furthermore, she has shown that in salivas of differing pH (pH 7.20 and 6.76) the morphological appearance of the degenerating PMN leukocytes is identical. Lymphocytes resemble those/

those seen in blood as do also monocytes, but eosinophils are also degenerate (Raeste, 1972). The morphologic appearance and size of PMN leukocytes in MNS generally corresponds to the changes observed in degenerating leukocytes of the blood (Koeffler, 1950). The degenerate state develops when the cells come in contact with saliva because those in the gingival fluid resemble those in the blood.

Kwaan and Astrup (1964) observe that PMN leukocytes contain a lysis producing protease but no activator of plasminogen, although Gans (1963) from his experiments with human, dog and rabbit leukocytes suggests an activator might be present. Astrup et al (1967) using plasminogen rich bovine plates and fibrinolytic autography demonstrates that washed intact leukocytes do not produce lysis although occasional weak activity is related to degenerate cells. Freeze-thawed PMN leukocytes lyse both plasminogen rich and plasminogen free substrates, from which result they conclude the lysis must be caused by a non-specific leukoprotease. Potassium thiocyanate extracts failed to demonstrate any measurable amount of stable activator.

However Goldstein et al (1971) (with Astrup) demonstrate normal viable human PMN leukocytes to contain small amounts of plasminogen activator, the release of which is enhanced by bacterial endotoxins. Wunschmann-Henderson et al (1972) report a comparative study of the fibrinolytic response to endotoxin of leukocytes. They found the number of fibrinolytically active cells increase following exposure to endotoxin (lipopolysaccharide B from Salmonella abortus) and approached the total leukocyte count. They also found during their work that complete destruction of leukocytes by freeze-thawing leaves the cells devoid of activator activity which may explain why in 1967, Astrup et al report no plasminogen activator in PMN leukocytes.

It appears then from the literature that PMN leukocytes contain small/

small quantities of plasminogen activator and larger amounts of a non-specific activator, a leukoprotease. The release of the specific activator is enhanced by endotoxin without apparently decreasing the number of viable leukocytes. Salivary leukocytes are in various stages of degeneration and exist in a fluid containing many bacteria which may or may not affect their plasminogen activator activity. It is necessary, therefore, to ascertain whether or not these salivary leukocytes have any specific activator activity upon human fibrin substrates. All the work of Astrup and his colleagues was performed upon bovine fibrin upon which MNS has no activator activity although such activity is consistently shown by MNS upon human fibrin, and so much of their work has been repeated here with human fibrin.

2. EXPERIMENT 1

Object: To study the plasminogen activator activity of leukocytes suspended in saline and MNS supernatant, and the effect of freeze-thawing on their activity.

Materials:

A. Collection of leukocytes.

1. Heparin: (20 i.u./ml.) diluted in saline 0.9% NaCl. from 25,000 i.u./ml. solution supplied by Weddel Pharmaceuticals Ltd., London.
2. 'Lomodex' 70: A sterile solution containing 6% w/v dextrans in 5% w/v Dextrose Injection B.P. (Fisons Ltd., England)
3. Blood: 10 mls. of freshly collected peripheral venous blood. To 8 mls. of heparin solution (20 i.u./ml.) were added 4 mls. of Lomodex 70. Into each of two sterile test tubes was put 5 mls. of this mixture. Ten mls. of peripheral venous blood, freshly collected, was divided evenly between the two tubes and gently mixed with the heparin-saline-dextrose (HSD) solution. After mixing the tubes were placed at an angle of 45^{o} in a $37^{o}C$ incubator for 30 - 40 minutes. The supernatant that formed was largely free from erythrocytes but contained leukocytes in suspension.

B. MNS supernatant.

Stimulated MNS (Methodology, p. XXVI) was collected from one of the laboratory staff who was in good health and who had no clinical evidence of gingivitis. The saliva was centrifuged at 20,000 r.p.m. (35,644 g.) for 30 minutes and the resulting supernatant stored at $4^{o}C$.

C. Fibrin films.

Fibrinolytic autography (Methodology, p. V)

D./

D. SHFP and SBFP with and without eACA (0.1 ml. M. eACA per plate).

E. Controls - tris buffer, pH 7.8, 0.15 M.
urokinase, 5 Ploug units/ml., pH 7.8, 0.15 M.
HSD
MNS supernatant.

Method:

The supernatant from both tubes of heparinised blood was removed, pooled, and gently rolled to resuspend evenly the leukocytes. Two mls. were then gently centrifuged (2 minutes at 800 - 1000 r.p.m.) and the resulting concentrated pellet of leukocytes washed three times in HSD. The cells were then resuspended in their original volume of saline and stored at 4^{o}C ('A').

A further 2 ml. of cells was removed and washed as above. After resuspension in their original volume of saline, the cells were subjected to rapid freeze-thawing for three times at -40^{o}C. After the final thaw, the cell suspension was stored at 4^{o}C ('B').

A final 2 ml. of cell suspension was removed and the cells washed as above. The final resuspension, however, was in the supernatant of MNS generated as described. These cells also were stored at 4^{o}C ('C').

Thus were prepared suspensions of washed leukocytes, viz.

A. Suspended in saline.

B. Suspended in saline and freeze-thawed.

C. Suspended in MNS supernatant.

A cell count was made of every suspension using an Improved Neubauer counting chamber. From each of these suspensions, the following procedures were carried out.

1./

1. Leishman stained films.
2. Fibrinolytic autography with the cells suspended in the fibrin.
3. Plating (3 x 0.03 ml.) upon SHFP and SBFP (with and without eACA) and incubation for 19 hours at 37°C. Controls were plated in accordance with Part 2, Experiment 3, Fig. 3.)

Results:

These are recorded in Tables 55 - 57 (Appendix 1).

Summary of Results:

A. Saline suspended leukocytes.

The concentration of washed leukocytes in saline ranged from 4870 - 250 per cu. mm.

For comparison, the upper limits of leukocyte counts in saliva recorded in the literature and discussed in the Introduction are :-

Healthy edentulous mouths	143 per cu. mm.
Healthy dentulous mouths	1,364 per cu. mm.
Chronically inflamed mouth	2,389 per cu. mm.

None of the suspensions produced lysis or probable lysis upon SHFP or SBFP either with or without eACA, and none showed lysis with fibrinolytic autography (Fig. 25)

B. Freeze-thawed leukocytes.

The concentration of freeze-thawed leukocytes in saline ranged from 35,000 - 288 per cu. mm.

The fibrinolytic activity of the suspensions can be described in three bands.

1. 35,000 - 16,000 per cu. mm. With one exception, all these concentrations displayed lysis upon SHFP and SBFP both with and without eACA. The eACA readings were consistently/

consistently lower with the human plates but very similar with the bovine plates.

2. 16,000 - 2,750 per cu. mm. Variable activity which is approximately proportional to concentration. The eACA readings are again consistently lower with the human plates, but very similar with the bovine.

3. 2,750 - 288 per cu. mm. With one exception (1600) none of these suspensions produced lysis upon SHFP or SBFP with or without eACA.

None of the suspensions showed lysis with fibrinolytic autography (Figs. 26 and 27.) although the cells showed varying degrees of disruption.

C. Washed leukocytes resuspended in the supernatant of MNS. The concentration of washed leukocytes resuspended in the supernatant of MNS ranged from 20,000 - 250 per cu. mm.

At 20,000 measurable lysis occurred upon SHFP both with and without eACA. There was no lysis of the SBFP either with or without eACA. From 18.000 - 2,250 occasional areas of probable lysis appeared on SHFP and SBFP, but only once on SHFP and SBFP with eACA.

Below a concentration of 2,250 per cu. mm., no lysis or probable lysis occurred at any time upon any plate.

None of the suspensions showed lysis with fibrinolytic autography (Figs. 28 & 29) although the cells showed varying degrees of disruption.

Discussion and Conclusions:

In the healthy dentulous mouth, an upper limit of leukocytes has been placed at about 1,364 per cu. mm. (Calonius, 1958). In this experiment washed leukocytes resuspended in the supernatant of MNS produced no lysis below a concentration of 2,250 per cu. mm. and no clear measurable lysis below 10,000 per cu. mm. and then the area produced was only 80 mm^2.

After freeze thawing, three times, no lysis upon SHFP occurred below a concentration of 2,750 per cu. mm. which is twice the concentration of the suggested upper limit.

Examination of the tables suggests there was some plasminogen activator present in addition to a leukoprotease as evidenced by the reduced lysis upon eACA plates, but the amount is very small and most of the activity is non-specific proteolysis.

It may be argued that the leukocytes resuspended in the supernatant of MNS would be no different than washed leukocytes which displayed no activity. However, cytological preparations of washed, freeze-thawed and MNS - resuspended leukocytes demonstrate the relatively normal appearance of the washed cells (Fig. 25) and the varying degrees of disruption associated both with freeze-thawing (Figs. 26 & 27) and the supernatant of MNS (Figs. 28 & 29).

Although the MNS supernatant (generated at 35,664 g.) used in this experiment was cell free, it might have retained in solution some endotoxin of salivary bacteria origin and indeed might contain rather more than in vivo since centrifugation at this force will tend to break up any cells left after the preliminary centrifugation at 4154 g. The complete absence of any fibrinolytic activity at cell concentrations commensurable with those of MNS is strongly suggestive that no endotoxin - leukocyte interaction takes place in MNS and that salivary leukocytes, at the concentrations normally present in healthy dentulous mouths, play no significant role in the plasminogen activator activity of MNS.

Astrup et al (1967) using bovine fibrinogen did achieve lysis with freeze-thawed leukocytes on their autographs both with and without eACA, thus demonstrating the presence of a protease. Astrup manages to achieve a satisfactory autograph using 0.7% bovine fibrinogen, which he prepares himself. Using commercially available/

Fig. 25.

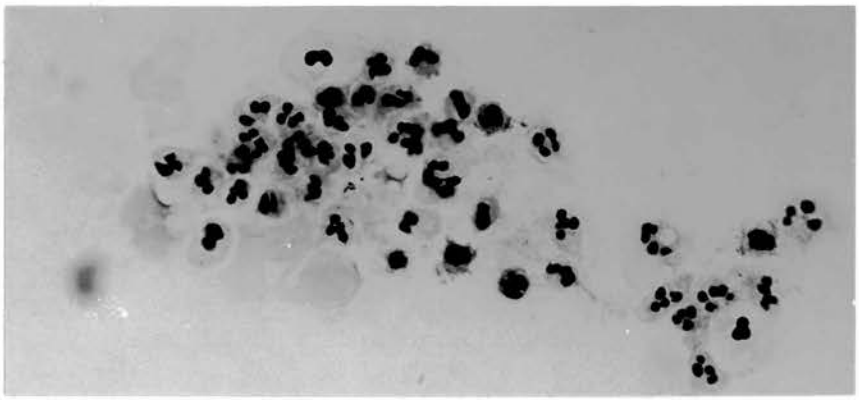


Fig. 26.

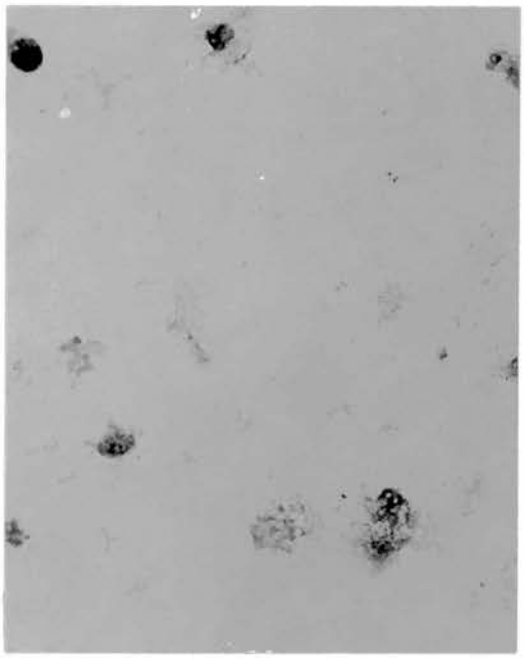


Fig. 27.

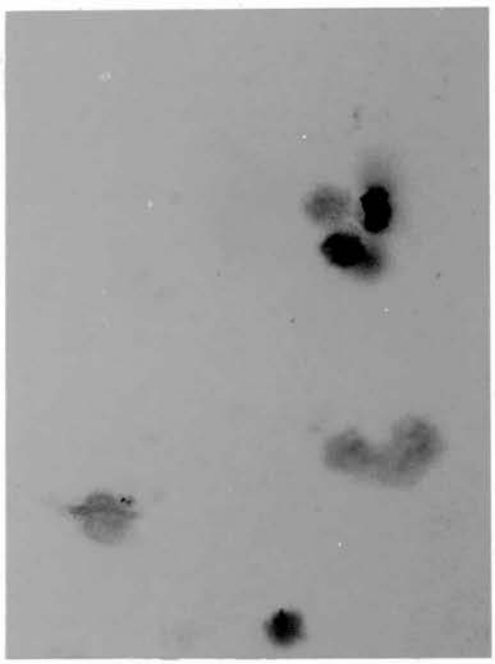


Fig. 25. Fibrinolytic autograph of suspension of leukocytes in saline showing no fibrinolysis. Human fibrin, incubated overnight at room temperature and 1 hour at 37°C. Mag. x 480.

Fig. 26. Smears of freeze-thawed suspensions of leukocytes in saline showing disrupted leukocytes. Stained with Leishman. Mag. x 480.

Fig. 27. Fibrinolytic autograph of freeze-thawed suspension of leukocytes in saline showing disrupted leukocytes but no lysis. Human fibrin, incubated at room temperature overnight and 1 hour at 37°C. Mag. x 480.

Fig. 28.

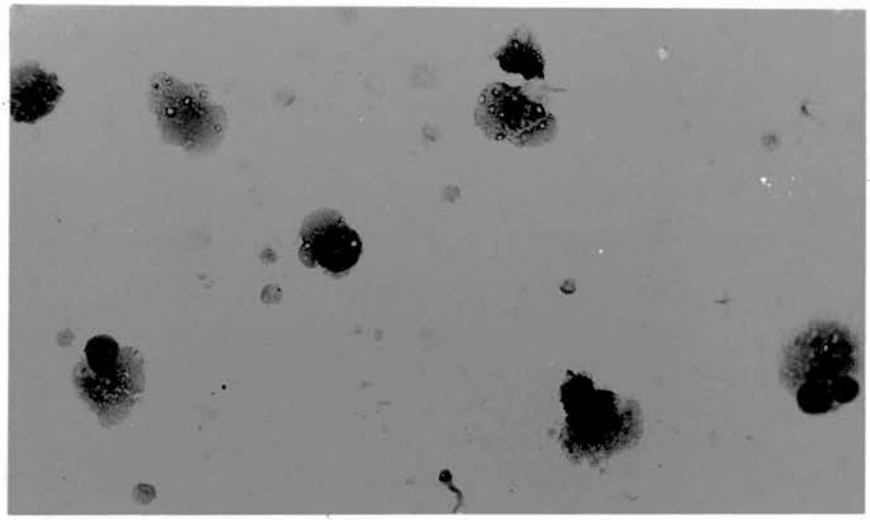


Fig. 28. Smears of leukocytes suspended in MNS supernatant showing disrupted leukocytes. Stained with Leishman. Mag. x 480.

Fig. 29.

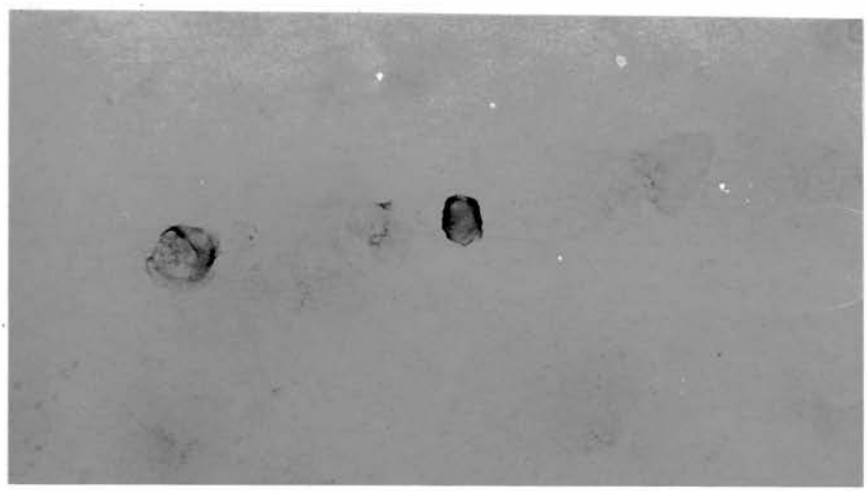


Fig. 29. Fibrinolytic autograph of leukocytes suspended in MNS supernatant showing disrupted leukocytes but no lysis. Human fibrin, incubated overnight at room temperature and 1 hour at 37°C. Mag. x 480.

able bovine fibrinogen, the writer and his colleagues in the Laboratory have never successfully produced a film at this concentration that was satisfactory and when such a dilution was approached, it would not adequately stain to demonstrate a differential between lysed and non-lysed areas. Wunschmann-Henderson et al (1972) using Astrup's techniques now find that smears prepared from frozen and thawed suspensions show no fibrinolytic cells on either plasminogen rich or plasminogen free fibrin. They conclude from their data that complete disruption of suspended cells leaves the stroma devoid of plasminogen activator although not necessarily a protease and thereby imply the presence of an activator they did not previously (1967) demonstrate. In 1972 they found that the leukoprotease activity of individual cells was too little to detect, although in 1967 this was what they found and illustrated.

In order to increase the sensitivity of their techniques, Astrup writes "... and the rinsing in tap water of the formaldehyde-fixed slides was extended to 20 hours to make the zones of weak lysis around the individual cells more visible." Given that he is starting with a very thin film, the danger of an artifact presenting as lysis is considerable. Evidence is presented in the next Chapter of this work of "cell shrinkage" during autography and its appearance as lysis. It cannot be stated that lysis by leukocytes using fibrinolytic autography cannot be achieved, but rather that this writer has been unable to reproduce satisfactorily the conditions under which Astrup has occasionally demonstrated fibrinolysis with freeze-thawed leukocytes.

3. EXPERIMENT 2

Object: To compare the plasminogen activator activity in MNS of a dentate population with that of an edentate population.

Materials:

1. Minimally stimulated MNS (Methodology, p. XXV) was collected from 20 male and 20 female edentulous adults who were attending the Prosthetic Department of Edinburgh Dental Hospital. The oral mucosa of each subject was examined for any evidence of oral ulceration or palatal stomatitis and only those persons in whom there was no clinical sign of stomatitis and who were in good health were asked to contribute.

2. SHFP. (Methodology, p. I).

3. Controls. Urokinase. Positive. 5 Ploug units/ml. pH 7.8, 0.15 M.
Tris buffer. Negative. pH 7.8, 0.15 M.

Method:

The samples of MNS were collected between 14.00 and 14.30 hours. Three 0.03 ml. drops of each saliva were set up on SHFP controlled as described in Part II, Experiment 3, Fig. 2 , and incubated at 37°C for 19 hours.

Results:

These are recorded in Table 58 (Appendix 1).

Summary of Results:

Average area of lysis (in sq. mm.) produced by 3 x 0.03 ml. drops of MNS upon SHFP after 19 hours incubation at 37°C.

	Male Edentates	Female Edentates
Mean (20)	201	197
S.D.	76	80

These results may be compared with those recorded in Part II, Experiment 5 where the lysis recorded is that produced by 50 male and 50 female dentates. Male dentates - Mean (50) 197. S.D. 100. Female dentates. Mean (50) 216. S.D. 109.

Discussion and Conclusion:

There is no significant difference between these two sets of figures. The important difference between the two populations in respect of the presence of leukocytes is, as discussed in the introduction to 'Salivary Leukocytes', their almost complete absence from the saliva in edentates with healthy mouths. As no significant difference exists between the two populations in respect of fibrinolytic activity, it seems highly improbable that salivary leukocytes could be making a major contribution to the plasminogen activator activity of MNS.

4. DISCUSSION AND CONCLUSION

It is apparent from the literature that PMN leukocytes contain small quantities of plasminogen activator and larger amounts of non-specific activator, a leukoprotease. It is also apparent that the release of activator is enhanced by certain endotoxins.

In the experimental work described above, no activator activity has been demonstrated in PMN leukocytes by fibrinolytic autography. However, using the fibrin plate technique, concentrations of leukocytes in excess of 2750 per cu. mm. when freeze-thawed brought about lysis upon SHFP, and leukocytes freshly harvested and resuspended in the supernatant of MNS also lysed SHFP but only caused measurable lysis when in excess of 10,000 per cu. mm. Fresh leukocytes resuspended in saline did not lyse SHFP. The results support the previous published evidence that there is some plasminogen activator present in addition to a leuko protease as evidenced by the reduced lysis upon eACA plates, but the amount is very small.

The lowest concentration of leukocytes producing lysis of SHFP occurred with the freeze-thawed preparations but even this concentration was about twice the upper limit of the concentration of leukocytes found in the healthy dentulous mouth. This finding, taken in consideration with the fact that no significant difference between dentate and edentate MNS could be found in respect of fibrinolytic activity, strongly suggests that although salivary leukocytes do have plasminogen activator activity, it constitutes a very minor component of the fibrinolytic system in MNS.

CHAPTER 6

ORAL EPITHELIAL CELLS

1. Oral epithelial cells.

2. Experiment 1. To study the fibrinolytic activity of oral epithelial cells by fibrinolytic autography.

3. Experiment 2. To study the fibrinolytic activity of oral epithelial cells using SHFP.

1. ORAL EPITHELIAL CELLS

Large numbers of desquamated epithelial cells are found in MNS. The number of epithelial cells in the saliva of ten adults (Deakins et al, 1941) ranged between 2620 and 18,500 per ml. Higher counts in the very young are paralleled by increased cell counts in edentulous persons (Calonius, 1958). These high counts may be due to the friction on the oral mucosa of foods and sucking which rubs off the cells (Klein, 1962).

Desquamated oral epithelial cells carry a variable burden of closely adherent bacteria, which in some cases may completely cover the surface (Bradley, 1948) and in a few cases may be within the cytoplasm (Montgomery, 1951).

These adherent bacteria are very resistant to being washed off and therefore when fibrinolytic studies of salivary epithelial cells are carried out, the bacteria are an unavoidable contaminant. Birn and Fejerskov (1971) and Wunschmann-Henderson and Astrup (1972) have described human oral epithelium as having fibrinolytic activity. Both studies used buccal smears examined by fibrinolytic autography. Birn and Fejerskov (1971) describe a "minority" of cells causing lysis, the cells being derived from all layers of the epithelium including anuclear superficial cells. Wunschmann-Henderson and Astrup (1972) describe up to 36% of cells as causing lysis. They found the superficial cell layers to be inactive, only the younger cells from the deeper layers of the epithelium being active, giving an inverse relationship between epithelial cell maturation and fibrinolysis. Unless the oral mucosa is inflamed or ulcerated, cells from the deeper layers of the epithelium are not present in saliva in large numbers. Surprisingly, fibrinolytic autography of frozen sections of human oral mucosa does not show lysis in relation to the epithelium (Harris and Pannell, 1973).

To/

To assess the importance of the fibrinolytic activity of epithelial cells in saliva, the fibrinolytic activity of naturally desquamated epithelial cells in salivary pellet, as well as the fibrinolytic activity of cells obtained from buccal, palatal and dorsal tongue smears was examined.

2. EXPERIMENT 1.

Object: To study the fibrinolytic activity of oral epithelial cells by fibrinolytic autography.

Materials:

1. Pellet. In excess of 12 ml. of MNS (stimulated flow, Methodology, p.XXVI) was collected from a healthy dentate with no evidence of gingivitis. The MNS was centrifuged at 4154 g. for 20 minutes at 4°C and the supernatant discarded leaving approximately 1 ml. of pellet.

2. Smears. After ensuring the subject had no gingivitis or throat infection, buccal, palatal and tongue smears were made by gently scraping the relevant tissue with wooden tongue spatulas and then suspending the scrapings in tris buffer (pH 7.8, 0.15 M). In order to obtain sufficient cells from the palate, it was necessary to pool scrapings.

3. Materials for human fibrinolytic autography (Methodology, p.V)

Method:

Pellet. From the fresh pellet, smears were made for autography and thereafter the pellet was divided into two. One half was snap frozen with carbon dioxide and five micron sections cut and placed on glass slides for autography. The other half was washed 4 times with 20 ml. volumes of tris buffer at 4°C by centrifugation at 4154 g. Smears were made from the washed pellet as above. For descriptive purposes, these preparations were called, pellet, frozen pellet and washed pellet respectively.

Smears. The cell suspensions prepared as above were centrifuged at 4154 g. for ten minutes at 4°C and the pellet generated washed three times with tris buffer and finally smeared onto glass slides for autography. /

autography.

The smears were labelled according to the cell type, e.g. buccal smears. Human fibrin films both with and without eACA were prepared for each smear or section. (Methodology, p. V).

Results:

The fibrin autographs of salivary pellet showed isolated foci of lysis (Fig. 30) which with prolonged incubation times became confluent (Fig. 31). When eACA was incorporated in the fibrin film, no lysis occurred even with a similar incubation time (Fig. 32) and this provided further evidence of the specificity of the fibrinolytic activity of MNS demonstrated in Part II.

When examined more closely, the cell in the centre of each focus of lysis could be seen under the microscope, but could only be demonstrated photographically by adjusting the exposure of the negative (Figs. 33 and 34). The active cells appeared to be both nucleated squamous epithelial cells (Figs. 35 and 36), and anuclear epithelial cell fragments (Figs. 37 and 38). Similar foci of lysis developed in smears from the cheek and tongue (Figs. 39 and 21). Because of technical difficulties, it was impossible to give a very accurate figure for the percentage of active cells, but not more than 2% of the buccal cells were active and the impression was that even fewer tongue cells were active. No lysis was seen associated with the keratinised cells in the palatal smears.

Discussion:

The frozen pellet sections clearly demonstrated foci of lysis but were too dense to accurately locate the source. The pellet smears washed and unwashed showed epithelial cells in the centres of the lytic foci. Bacteria were also present on the same preparations unrelated to the foci of lysis and it is, therefore, unlikely that the bacteria attached to the epithelial cells were responsible for the lysis although the possibility/

Fig. 30.

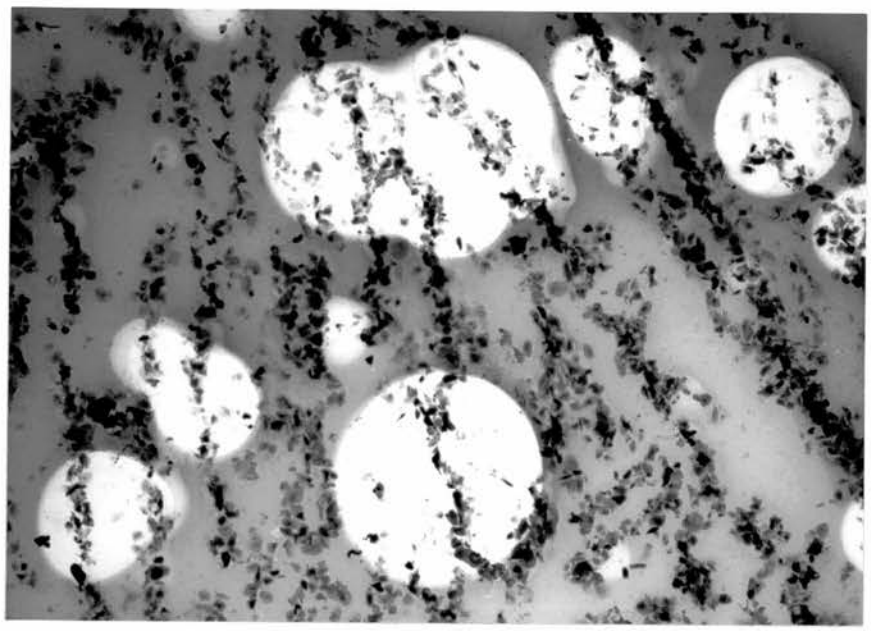


Fig. 31.

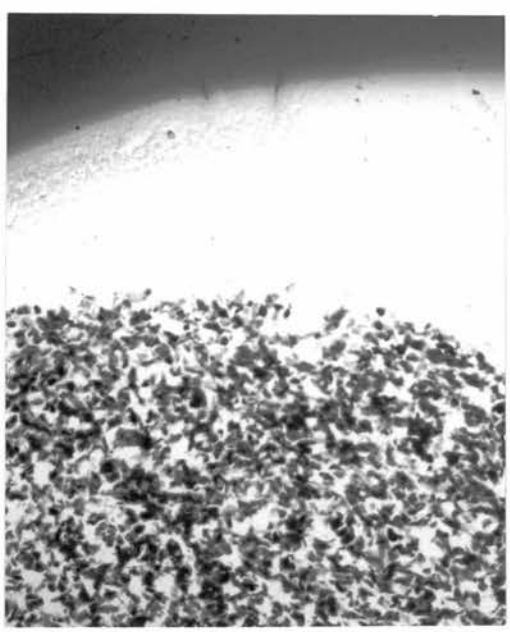


Fig. 32.

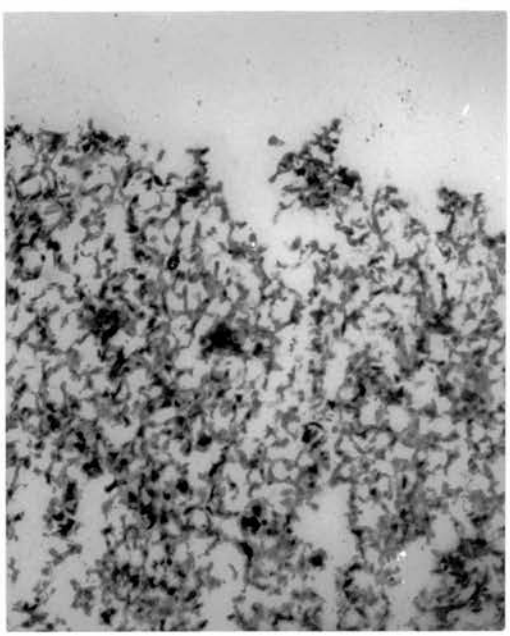


Fig. 30. Fibrinolytic autograph of a frozen section of MNS pellet showing isolated foci of lysis, the larger foci becoming confluent but with small foci of lysis also present. Human fibrin, incubated overnight at room temperature and for 1 hour at 37°C. Mag. x 30.

Fig. 31. Fibrinolytic autograph of a frozen section of MNS pellet showing confluent lysis. Human fibrin, incubated 18 hours at 37°C. Mag. x 30.

Fig. 32. Fibrinolytic autograph of serial section to Fig. 31 showing no lysis when eACA was incorporated in the fibrin film. Human fibrin, incubated for 18 hours at 37°C. Mag. x 30.

Fig. 33.

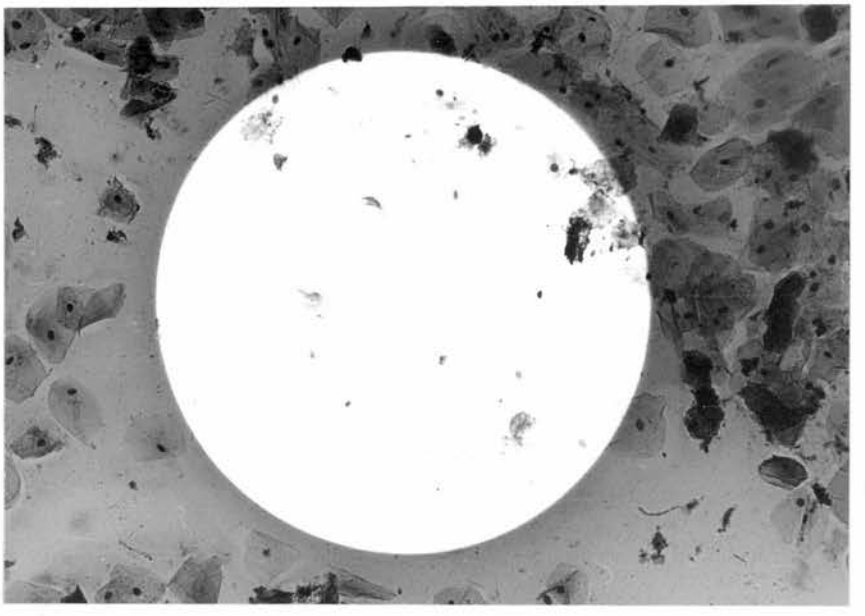
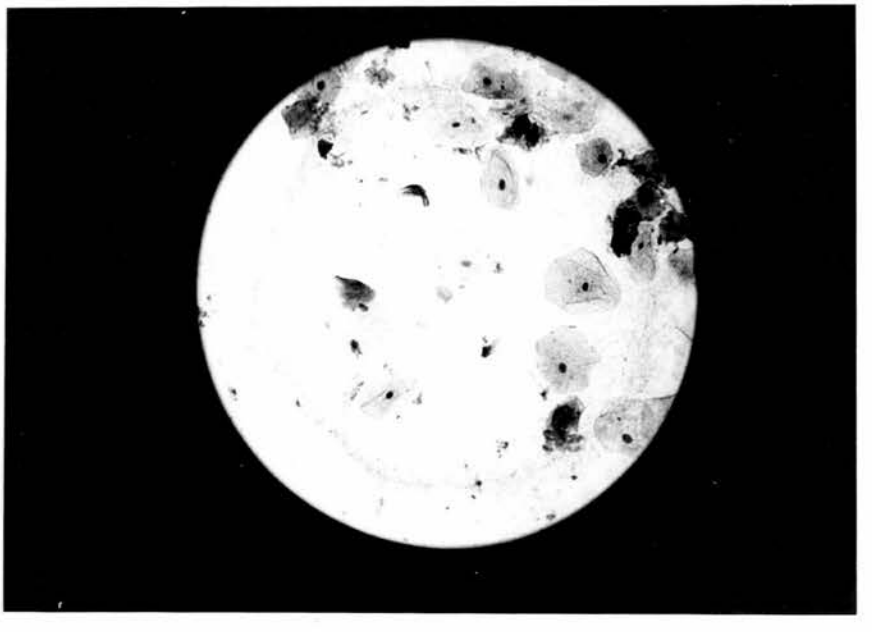


Fig. 34.



Figs. 33 & 34. Fibrinolytic autograph of a smear of a pellet (MNS) showing a well defined focus of lysis , which when the negative was over exposed shows an anuclear cell in the centre. Human fibrin , incubated overnight at room temperature and 1 hour at 37^{o}C. Mag. x 48.

Fig. 35.

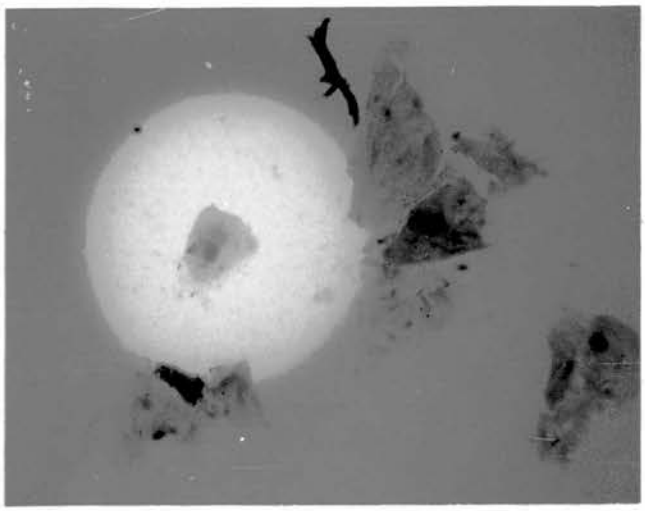
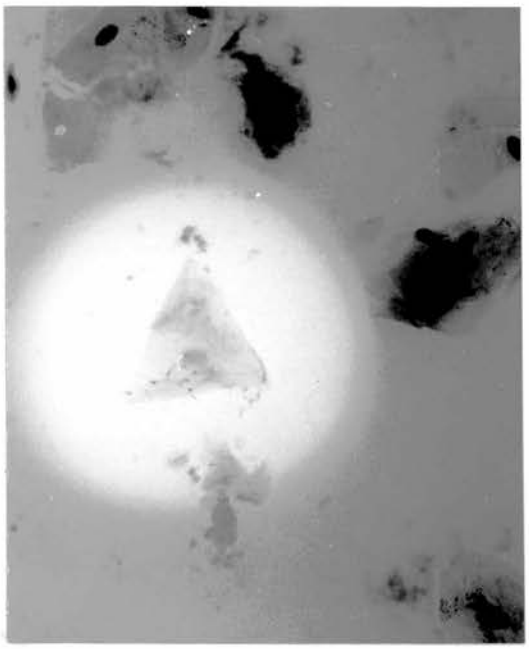


Fig. 36.



Figs. 35 & 36.

Fibrinolytic autographs of smear of MNS pellet showing nucleated squamous epithelial cells in the centres of the foci of lysis. Human fibrin, incubated overnight at room temperature and 2 hours at 37°C. Mag. x 300.

Fig. 37.

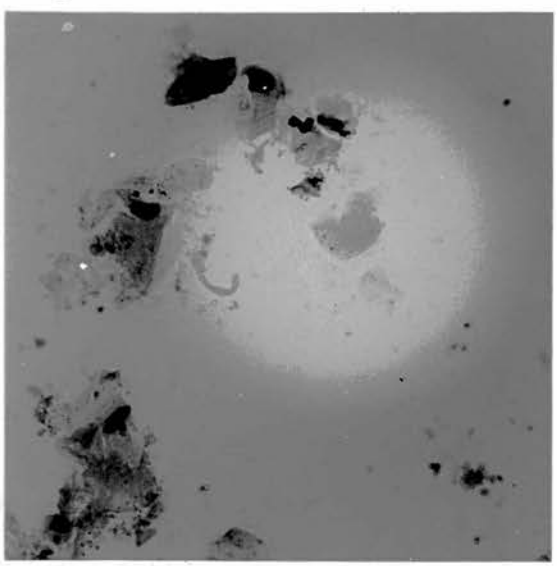
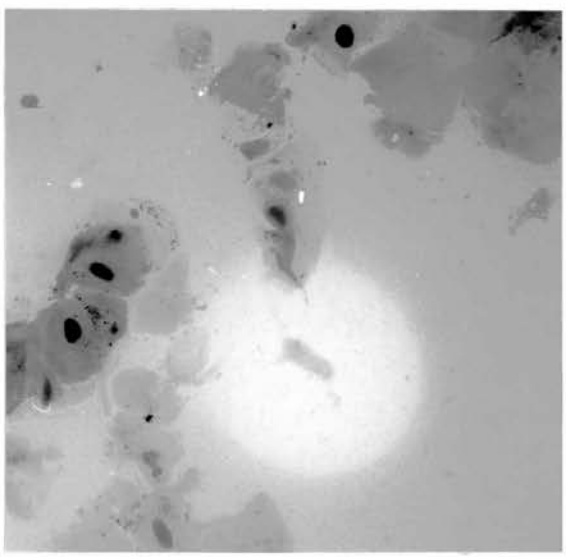


Fig. 38.



Figs. 37 & 38.

Fibrinolytic autograph of smear of MNS pellet showing anuclear epithelial cell fragment in the centres of the foci of lysis. Human fibrin, incubated overnight at room temperature and 2 hours at 37°C. Mag. Fig. 37 x 120. Fig. 38 x 300.

Fig. 39.

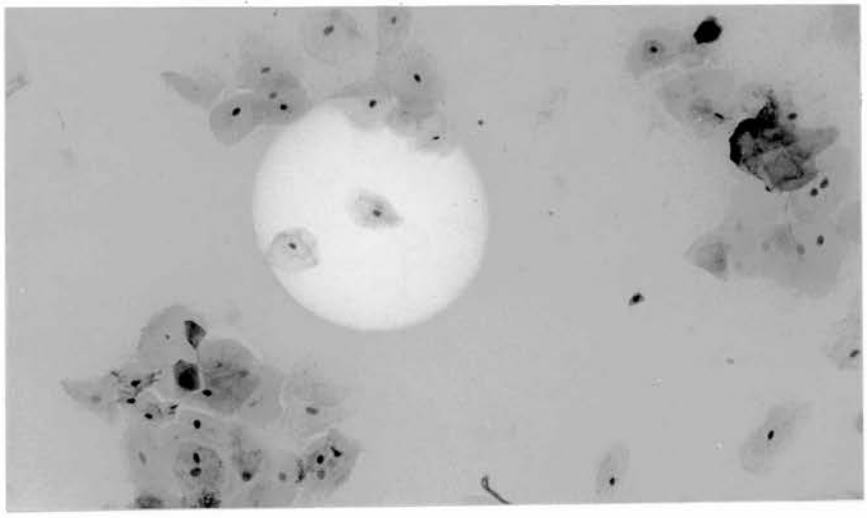


Fig. 39. Fibrinolytic autograph of a buccal smear showing lysis related to a nucleated squamous epithelial cell. Human fibrin , incubated overnight at room temperature and 1 hour at 37°C. Mag. x 120.

Fig. 40.

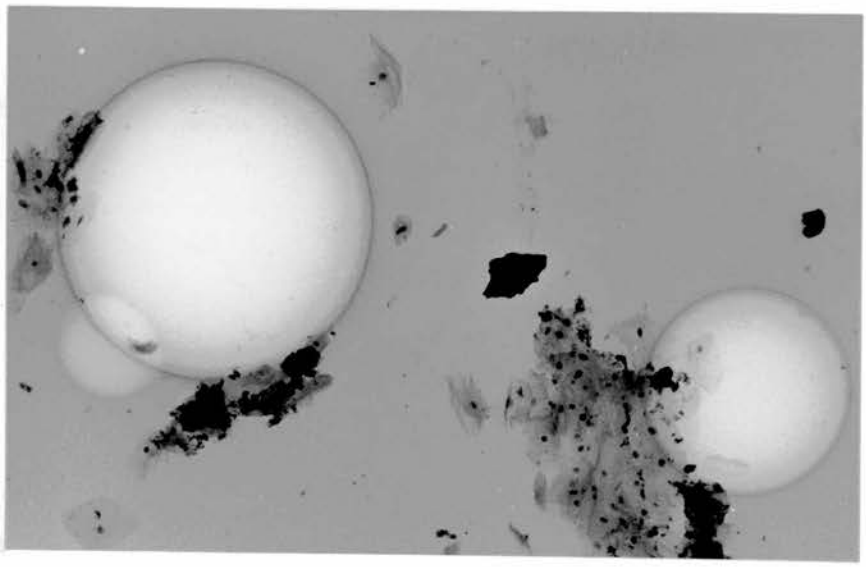


Fig. 40. Fibrinolytic autograph of a buccal smear with bubbles made in the fibrin. The fibrin was not incubated before fixation and no cells are present in the centres of the clear circles. Human fibrin. Mag. x 120.

possibility that they might enhance the epithelial cell activity cannot be ruled out since Wunschmann-Henderson et al (1972) have drawn attention to endotoxin induced fibrinolytic activity in suspensions of leukocytes. The writer was unable to determine the degree of maturity of the pellet cells using Papanicolaou's staining method because the fibrinolytic autographs were too thick. The writer was unable to prepare adequate human fibrin films using a concentration of fibrinogen less than 2% while Wunschmann-Henderson and Astrup achieve a film using 0.7% bovine fibrinogen.

Fibrinolytic activity was shown by approximately 2% of the buccal cells in any one smear and by lesser numbers of cells from the tongue. This is consistent with Birn and Fejerskov (1971) who describe occasional cells causing lysis but compares unfavourably with Wunschmann-Henderson and Astrup (1972) who record activity associated with up to 36% of buccal cells. The latters films are much thinner than the writers, the fibrinogen solution used is 0.75% and made from plasminogen rich bovine fibrinogen and, furthermore, the autographs are washed in water after fixation to increase the visibility of lysis, a most hazardous procedure (Chapter 5). The writer has observed several hundred autograph preparations and has found that there is a real danger of misinterpreting artefacts and wonders whether such artefacts might partly account for the high percentage of fibrinolytically active cells described by Wunschmann-Henderson and Astrup (1972).

Two main types of artefact have been noticed. One was an air bubble in the film which was distinguished by a well marked line (sometimes double) around the periphery (Fig. 40). A clear focus of lysis sometimes had a similar line but not as well marked. The other artefact was a form of distortion where a well delineated clear area appeared around the periphery of the cell which was not circular, and the size of which was not related to incubation time (Figs. 41 and 42). Such areas/

Fig. 41.

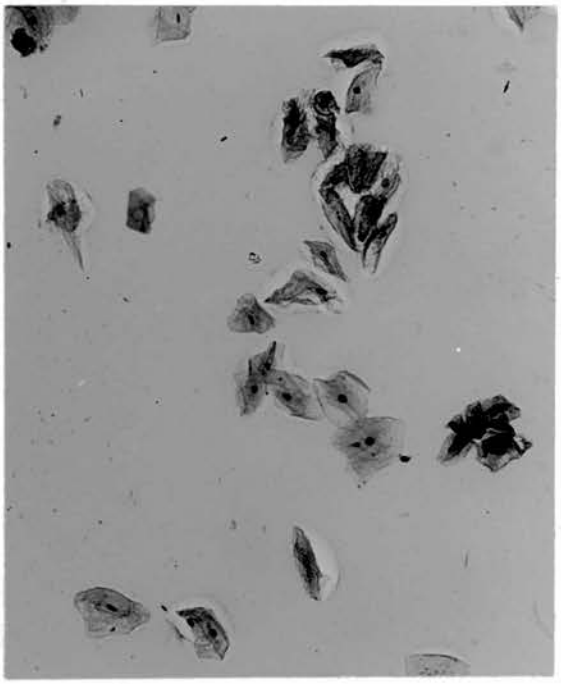
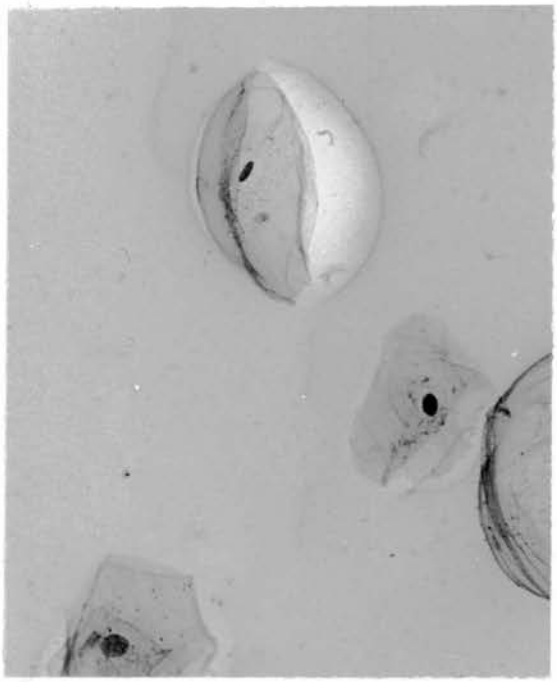


Fig. 42



Fibrinolytic autographs of buccal smear showing clear areas around the cells which are not circular and the development of which was not related to incubation times. Human fibrin, incubated at 37°C. for 2 hours. Mag. Fig. 41 x 48. Fig. 42 x 300.

Fig. 43.

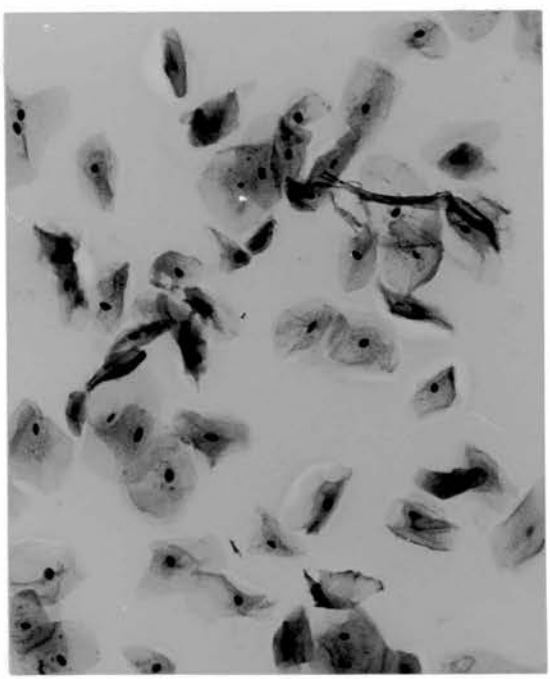
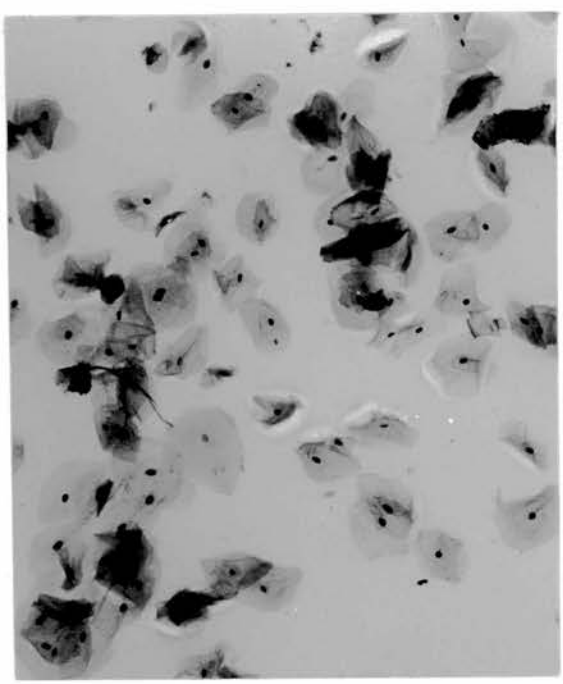


Fig. 44.



Figs. 43 & 44.

Fibrinolytic autographs of buccal cells with eACA in the fibrin showing similar clear areas around the cells to those seen in Figs. 41 & 42. Human fibrin, incubated overnight at room temperature. Mag. Fig. 43 x 150. Fig. 44 x 120.

areas were also seen when eACA was included in the fibrin (Figs. 43 and 44). This latter type of artefact was particularly apparent towards the edges of films from where contraction might have occurred during polymerisation of the fibrin. However, deliberate attempts to increase the incidence of this artefact by preparing thinner films failed. It was frequently related to apparently folded and misshapen cells, and so might be due to changes in the cell per se, possibly accentuated by fibrin contraction moving the cells during polymerisation.

Conclusions:

1. Spontaneously desquamated epithelial cells in saliva show fibrinolytic activity when examined by fibrinolytic autography, both nucleated cells and anuclear cells and fragments showing activity.

2. Approximately 2% of artificially desquamated buccal epithelial cells show fibrinolytic activity.

3. EXPERIMENT 2.

Object: To study the fibrinolytic activity of oral epithelial cells using SHFP.

Materials:

1. Washed pellet. 12 ml. of MNS was collected by stimulated flow (Methodology, p. XXVI) from each of 20 persons who were in good health and who had no evidence of gingivitis. Each MNS sample was centrifuged at 4154 g. for ten minutes at $4^{o}C$ after which the supernatant was discarded and the pellet washed four times by centrifugation also at 4154 g. with tris buffer. The pellet was finally re-suspended in 0.75 ml. of tris buffer which would represent a cell concentration of 16x that of the original MNS provided there was no cell loss. The concentrated pellet was considered to be at a concentration of 100% and serial dilutions of 50, 25 and 12.5% were prepared from it using tris buffer as dilutant. A count was carried out upon each 100% concentrated sample.

2. Cell suspensions. Using wooden tongue spatulas buccal, palatal and tongue cells were gently scraped from their respective sites and collected in tris buffer. The suspensions of each cell type were pooled to ensure counts in excess of 10^{3}/ml. Each suspension then was washed as above. After washing, a cell count was made of each specimen.

3. SHFP (Methodology, p. (Methodology, p. I).

4. Counting chamber. Improved Neubauer.

5. Controls. Positive. Urokinase. 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M. Negative. Tris buffer, pH 7.8, 0.15 M.

Method:/

Method:

Concentrated and serially diluted pellet and the three cell suspensions were plated (3 x 0.03 ml.) upon SHFP and incubated for 19 hours at 37°C. Controls were plated as in Part II, Experiment 3, Fig. 3.

Results:

These are recorded in Tables 59 and 60 (Appendix 1).

Summary of Results:

Salivary pellet. The epithelial cell count, if the cells had been re-suspended in their original volume of saliva gave a mean figure of 16,578. S.D. 3,248 per cu. ml. In the concentrated pellet, the mean cell count was 265,000. S.D. 52,239 per cu. ml.

Lysis was reduced with decreased cell concentration, the mean area lysed at each concentration being 158, 141, 117 and 100 sq. mm. respectively.

Cell suspensions. Fibrinolytic activity decreases with reduced cell concentration with a suggestion that the buccal epithelial cells are the more active.

Discussion and Conclusion:

The conclusions are as stated above and are considered in the general discussion.

CHAPTER 7

Object: To examine the relationship between the components of MNS in generating plasminogen activator activity.

Comment:

This experiment was conducted after work on proactivator (Part V) had established the presence of plasminogen in saliva. It is included here in order to maintain continuity in the activator discussion.

Materials:

1. MNS. Stimulated MNS (Methodology, p. XXVI) was collected from twenty persons who were in good health, were taking no drugs, and who had no clinical evidence of gingivitis.

2. Centrifuge. Universal Junion IKS.

3. Millipore Filter system. Millipore GS 0.22 micron 25 mm. filter supported in a Micro-syringe, Luer inlet, 25 mm.

4. Plasminogen. Lyophilized. 25 caseinolytic units (C.U.) A.B. Kabi, Sweden.

5. Fibrin plates. SHFP. (Methodology, p.I).

6. Control solutions. Urokinase. Positive. 5 Ploug units/ml. in tris, pH 7.8, 0.15 M.
Tris buffer. Negative. pH 7.8, 0.15 M.
Tris/plasminogen solution. Concentration of plasminogen, 0.05 CU/ml.

Method:

A minimum of 11 mls. of MNS was collected from each subject. After vortex stirring, 0.5 mls. was removed and stored at 4^{o}C. This sample was representative of the whole MNS (A).

From/

From the remaining MNS, 10 mls. was removed and centrifuged at 4154 g. for thirty minutes at 4°C. 8 mls. of supernatant was then removed and the pellet resuspended in the remaining 2 mls. of fluid.

From the 8 mls. of supernatant, 2 mls. was removed (B) and stored at 4°C. The remaining 5 ml. of supernatant was then filtered through a 0.22 micron millipore filter. From the filtrate obtained, 2 mls. was removed (C), and stored at 4°C.

The 2 mls. of resuspended pellet was then washed ten times in tris buffer, for ten minutes at 4154 g. The washing volume was 20 ml. After the final wash and centrifugation, the pellet was once more resuspended in 2 mls. of tris buffer and then divided into four 0.5 ml. aliquots. One of these was stored at 4°C (D).

The remaining three were centrifuged for ten minutes at 4154 g. after which the supernatant was removed. To one was added 0.5 ml. of MNS supernatant (E). To one was added 0.5 ml. of MNS supernatant (E). To another was added 0.5 ml. of filtered supernatant (F). To the last was added 0.5 ml. of the tris/plasminogen solution (0.05 CU/ml.)(G). (The supernatant and filtered supernatant added here were taken from the samples B and C respectively).

There were then seven test solutions.

A. MNS.

B. Low speed supernatant of MNS.

C. Filtered supernatant of the MNS.

D. Washed pellet resuspended in tris buffer.

E. Washed pellet resuspended in supernatant of MNS.

F. Washed pellet resuspended in filtered supernatant of MNS.

G. Washed pellet resuspended in a tris/plasminogen solution of a strength equal to that of plasminogen in the average saliva (0.05 CU/ml.)

Each of these samples was vortex stirred to resuspend evenly its constituents/

constituents and then plated 3 x 0.03 ml. upon SHFP. Controls were as illustrated in Part II, Experiment 3, Fig. 3.

Results:

These are recorded in Table 61 (Appendix 1).

Summary of Results:

A.	MNS.	20	lysis.
B.	Sup./MNS.	17	lysis.
		2	probable lysis.
		1	no lysis.
C.	Filt./sup.	10	lysis.
		6	probable lysis.
		4	no lysis.
D.	Pellet/tris.	7	lysis.
		8	probable lysis.
		5	no lysis.
E.	Pellet/sup.	15	lysis.
		5	probable lysis.
F.	Pellet/filt. sup.	18	lysis.
		2	probable lysis.
G.	Pellet/tris and plasminogen solution	19	lysis.
		1	probable lysis.

Discussion:

This was a very artificially contrived experiment designed to be indicative rather than quantitative.

There are two major objections :-

1. The mucus in MNS prevented the completely even suspension of particulate matter.
2. There would be a loss of cells and especially cell fragments in/

in the washing of the pellet. Furthermore, the cell composition must have undergone an alteration in relative composition such that epithelial cells would be proportionately increased relative to the much smaller leukocytes and bacteria, some of which would have been lost in the washings. For these two reasons, the washed pellet cannot be simply regarded as the cells found in saliva without the salivary secretions.

Nevertheless, an examination of the results is interesting.

The results can be considered in two groups :- A - C and D - G.

A - C:

The results here are consistent with those recorded in Part III, Experiment 3.

A. All samples show lysis. (Experiment 3, all samples showed lysis).

B. Evidence of lysis in 19/20 samples. (Experiment 3, all samples showed lysis).
Activity reduced in 18/20 samples. (Experiment 3, reduced in 34/40 samples).

C. Evidence of lysis in 16/20 samples. (Experiment 3, 26/40 samples).

D - G:

There is little difference between the results for E and F. D and G are very interesting. D consisted of thoroughly washed cells resuspended in tris and in a concentration (if no cells had been lost with washing) 5x greater than in whole MNS. There was evidence of lysis in 15/20 of the samples but lysis in only seven. The activator activity then of this suspension was very low.

G consisted of thoroughly washed cells suspended in a tris and plasminogen/

minogen solution which was prepared to a strength equivalent to that calculated for plasminogen in saliva. This then was the strength of plasminogen (approximately) also in E and F. There was little difference between D and G. (Mean values, and hence tests of significance cannot be made due to the presence of probable lysis.)

The plasminogen solution (0.05 CU/ml.) applied to all human plates upon which solution G was being tested were negative. The only difference between D and G was the presence of plasminogen in solution. D and G together strongly suggest the ability of salivary pellet cells to activate plasminogen and to produce lysis in the same order of magnitude as that produced by incubating similarly washed cells with the supernatant of saliva. This is indicative (no more) that the salivary plasminogen activator was derived from cells and these together with their derived fragments in the presence of plasminogen can provide the great majority of the total plasminogen activator activity of MNS.

Conclusion:

Results are presented that confirm the findings of the preceding experiments in Part III and IV. A solution of plasminogen in tris buffer prepared at the same concentration as that found in the average saliva was found to produce no lysis upon an S HFP when applied alone, but when used to suspend washed salivary pellet cells, predominantly epithelial cells, produce lysis similar to that produced by suspending the cells in the supernatant of MNS. The washed cells suspended in tris buffer alone also produced evidence of lysis, but of a considerably smaller magnitude. These findings are interpreted as providing further evidence for the cell associated activator activity of the salivary pellet.

CHAPTER 8

DISCUSSIONS AND CONCLUSIONS

It has been shown that whole salivary pellet has plasminogen activitator activity and that it is highly improbable that any of this activity is contributed by salivary mucus, bacteria or leukocytes. In Part III, evidence was presented that strongly indicated the absence of a soluble activator. The only remaining major component of the pellet is desquamated epithelial cells.

Evidence has been presented in this Part confirming the findings of Birn and Fejerskov (1971) and Wunschmann-Henderson and Astrup (1972) that some oral epithelial cells have activator activity. In preferring to use human fibrinogen for the preparation of fibrinolytic autographs, it has not been possible to stain the cells with Papanicolaou's technique since the thickness of the human film prevents adequate penetration of the stains and, therefore, no comment can be made upon the degree of cornification of the active cells. Up to 2% of the cells were active and this compares unfavourably with the figure of 36% found in non-smokers by Wunschmann-Henderson and Astrup (1972). This matter has been discussed more fully in the section concerned with autography.

It has also been clearly shown that washing of the pellet results in a considerable loss of activity which in view of what has been said concerning non-active components, must most probably reflect a loss of epithelial cells or cell fragments. The whole epithelial cell is very large and it is unlikely that many of them are lost during washing. However, in the density gradient experiment, the least dense fractions were also fibrinolytically active and photographic evidence was presented showing the presence of epithelial cell fragments and bacteria. With the strong evidence that bacteria play little/

little part in the fibrinolytic activity of saliva, this suggests that it was the epithelial cell fragments that were responsible. This is further indicated by the results of fibrinolytic autography and photographs have been presented illustrating epithelial cells in the centre of foci of lysis.

In Part III, it was demonstrated that after low speed centrifugation, 4154 g., the supernatant of MNS was still showing evidence of activator activity but that after high speed centrifugation, 35,664 g., this activity was removed completely not even being suggested upon SHFP after concentrating 25x. However, pellet was collected from twenty separate high speed centrifugations and tested for activator activity. All twenty samples were active and photographic evidence of the nature of the pellet was presented displaying once again a predominance of epithelial cell fragments.

Therefore from the evidence provided by the salivary pellet generated at high speed centrifugation, fibrinolytic autography and density gradient separation, the writer concludes that plasminogen activator activity is associated with cell fragments of epithelial cell origin. This deduction must be seen in context since bacteria were present in all three preparations described above, but the bacteriological experiments failed to provide any convincing evidence of activator activity that could be detected within the sensitivity of the systems used here whilst epithelial cells have been clearly indicted. Of course, the activator activity is not confined to epithelial cell fragments, fibrin plate experiments and fibrinolytic autography with washed pellet and buccal epithelial cell suspensions have demonstrated intact cell activity. The reconstitution experiment (Chapter 7) demonstrated the ability of washed epithelial cells to activate plasminogen. The findings with whole cells are consistent with the findings of Birn and Fejerskov (1971) and Wunschmann-Henderson and Astrup (1972). One specifically contradictory statement occurs in the literature. In the paper written by Wunschmann-Henderson/

Henderson and Astrup (1972) there appears this paragraph :-

"A possible correlation of cellular fibrinolytic activity with the fibrinolytic activity in saliva was studied by collecting samples of saliva from two non-smokers over a period of five weeks, yielding twenty-five samples from each individual. After centrifugation for thirty minutes at 1000 g. the fibrinolytic activity was determined by placing 0.025 ml. of the clear supernatant on preformed fibrin slides which were then incubated for 120 minutes at 37°C. The sediment was resuspended in saline to half the original volume and the number of epithelial cells and leukocytes was determined by standard procedures. The presence of bacteria or cellular debris was also recorded".

Nowhere in the paper does a statement of lysis associated with any of the above samples occur. There is no statement of the cell counts made or indeed any result produced at all. However, in the 'Discussion' there occurs this sentence, "Cellular fibrinolytic activity was not correlated with the fibrinolytic activity of the saliva." In their section 'Results', General Observations, they state "As previously reported (Albrechtsen and Thaysen, 1955) human saliva showed only traces of fibrinolytic activity, unrelated to the number of free epithelial cells, leukocytes, bacteria, amounts of debris or the fibrinolytic activity of corresponding buccal smears." This statement is misleading as it can be construed as suggesting that Albrechtsen and Thaysen examined a relationship between the fibrinolytic activity of saliva and salivary cells etc. They did not. Not a single word about cells occurs in their paper. The writer believes that Wunschmann-Henderson and Astrup (1972) do not mean to be misleading, but are agreeing with Albrechtsen and Thaysen (1955) in so far as to state that there are only traces of fibrinolytic activity in saliva. After centrifuging for thirty minutes at 4154 g., the writer has presented evidence of a trace of activity and this is surely consistent with Wunschmann/

Wunschmann-Henderson and Astrup's findings of a trace of activity after centrifuging for thirty minutes at only 1000 g. Had they subjected the supernatant to high speed centrifugation and examined the subsequent supernatant generated, they might have found no activator in solution and the necessity therefore to find an alternative source.

There remains the question of from where in the epithelial cell, the plasminogen activator activity arises. The writer has made no original investigations into this problem. Lack and Ali (1964) and Tice and Worth (1968) have presented evidence suggesting that the source of cellular fibrinolytic activity is the lysosomes and therefore one might expect that all cells containing lysosomes would be fibrinolytically active, i.e. probably all the epithelial cells, but it is known that plasminogen activators responsible for the local fibrinolytic activity are firmly attached to the structural proteins and not liberated under normal conditions (Albrechtsen, 1958). This type of activator is liberated only after injury to the cell (Ungar, 1952: Astrup, 1956). However, when it is liberated as may happen under the conditions of fibrinolytic autography, the amount may be very small, just enough to generate some plasmin and bring about focal lysis. It could also be labile once released, although stable within the cell. The writer at one stage collected 110 ml. of salivary pellet (equivalent to about 1 litre of MNS) homogenised it manually, subjected it to freeze-thawing and sonication and then attempted a fractionation of the supernatant of this "soup" on a G200 sephadex column. High speed centrifugation had not been applied to the supernatant and it therefore displayed a little activity but on fractionation, no activity emerged that could be measured upon SHFP. This was a crude attempt to extract an activator and characterize its molecular weight but it failed and a more refined approach to the problem will have to be evolved. Potassium thiocyanate extraction and further cell disintegration with separation of particles by centrifugation are but two possible lines of enquiry.

Conclusion :/

Conclusion:

The activator activity of human mixed native saliva is associated with epithelial cell and epithelial cell fragments. The source of the activator within the cell and cell fragments has not been elucidated.

PART V

PROACTIVATOR

of MNS using Sephadex G200 and examination for plasminogen in the fractions.

Chapter 3. Streptokinase-activated proactivator activity of the cellular components of MNS using SBFP and fibrinolytic autography.

Chapter 4. Discussion and Conclusions.

CHAPTER 1

Streptokinase-activated Proactivator Activity in Saliva

The nature of proactivator has been discussed in Part 1. It was concluded that most streptokinase-activated proactivator is plasminogen. (No attempt will be made in this work to determine the presence or absence of a proactivator that is not activated by streptokinase).

It is established that the fibrinolytic activity of saliva, mixed native, parotid and submandibular/sublingual is considerably enhanced with the addition of streptokinase (Albrechtsen and Thaysen, 1955: Schulte 1965: Nitta et al, 1967 and Tortelli, 1967). The only known biochemical effect of streptokinase is to bring about the conversion of human plasminogen to plasmin (McNicol and Douglas, 1972). Thus it seems probable that salivary proactivator is plasminogen.

Plasminogen has not been demonstrated directly in saliva. Albrechtsen and Thaysen (1955) and Taylor et al (1964) were unable to demonstrate its presence and claim its absence from saliva. Nitta et al (1967), using standard bovine fibrin plates and heated bovine fibrin plates demonstrate streptokinase-activated fibrinolytic activity upon both plates when saliva was mixed with 2,500 units/ml. streptokinase in a 1:1 v/v ratio. They conclude from these results the "presence of large amounts of proactivator and small amounts of plasminogen" in saliva. Gustafsson and Nilsson (1961) used the same technique to demonstrate the presence of plasminogen in gingival fluid. From these indirect experiments, there emerges the suggestion that plasminogen may be present in MNS and could be derived in part at least from the gingival fluid.

EXPERIMENT 1.

Object: To obtain an estimate of the normal range of streptokinase-activated proactivator activity in MNS.

Comment:

This experiment was conducted with the same specimens of MNS as Part II, Experiment 5., but no direct comparison between results obtained upon SHFP and SBFP can be made.

This is because :-

1. The sensitivity of the two types of plates is different (Part II, Experiment 2.)
2. The writer has been unable to record lysis of an SBFP by MNS without the addition of streptokinase. All MNS samples collected have shown activity upon SHFP which suggests some other factor, perhaps species difference, is operating in addition to sensitivity.
3. MNS is combined with streptokinase in the ratio of 1:1 in the following experiment and is, therefore, diluted whilst the results of MNS upon human plates record undiluted saliva.

Materials:

1. MNS samples. These were collected by minimal stimulation (Methodology, p. XXV) from 50 male and 50 female subjects aged 18 - 30 who were not taking drugs and who had no clinical evidence of gingivitis.
2. SBFP. (Methodology, p. IV).
3. Streptokinase, 2500 units/ml. in tris buffer, pH 7.8, 0.15M.

4./

4. Controls. Urokinase. Positive. 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
   Tris buffer. Negative. pH 7.8, 0.15M.
   Streptokinase (SK) - as above.

Method:

From each MNS sample stored at 4°C in the refrigerator, 0.5 ml. was removed and mixed 1:1 (v/v) with streptokinase.
This mixture was vortex stirred and plated 3 x 0.03 ml. upon SBFP. Control solutions were plated as described in Part II, Experiment 3, Fig. 2. All the plates were then incubated for 19 hours at 37°C.

Results:

These are recorded in Tables 62 and 63 (Appendix 1).

Summary of Results:

Average areas lysed in sq. mm. by 3 x 0.03 ml. of MNS/SK upon SBFP after 19 hours incubation at 37°C.

	Male	Female	Total
Mean	167 ± 46 S.D.	162 ± 51	164.4 ± 48

All samples were plated upon SBFP without the addition of streptokinase and no lysis was recorded with any sample. Streptokinase applied to the SBFP alone never produced lysis. All controls were valid.

Conclusions:

1. MNS contains a streptokinase-activated proactivator.
2. The range of streptokinase induced activity is considerable, 90 - 333 sq. mm.
3. There was no significant difference between the streptokinase-activated proactivator activity of MNS from males and females.

EXPERIMENT 2.

Object: To compare the streptokinase-activated proactivator activity of mixed native, parotid and submandibular/sublingual saliva.

Materials:

1. This experiment was carried out in conjunction with Part III, Experiment 1 using the same samples of MNS, parotid and submandibular/sublingual saliva.
2. SBFP. (Methodology, p. IV).
3. Streptokinase, 2500 units/ml. in tris buffer, pH 7.8, 0.15 M.
4. Controls: Urokinase, positive, 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
   Tris buffer, negative, pH 7.8, 0.15M.
   Streptokinase - as above.

Method:

Collection of salivas as described in Part III, Experiment 1. Each saliva was plated 3 x 0.03 ml. upon SHFP and SBFP. From the remaining saliva in each sample 0.5 ml. was removed, mixed 1:1 (v/v) with streptokinase, and plated 3 x 0.03 ml. upon SBFP. Controls were set up as described in Part III, Experiment 1, but in addition 2 x 0.03 ml. drops of streptokinase were placed upon each SBFP.
All the plates were then incubated at 37^{o}C for 19 hours.

Results:

These are recorded in Tables 64 and 65 (Appendix 1).

Summary/

Summary of Results:

Subjects		Average Areas of Lysis in Sq. Mm.		
		MNS	Parotid	Submandibular/ sublingual
Male	Mean (20)	181	99	91
	S. D.	64	28	17
Female	Mean (20)	165	111	99
	S. D.	63	28	24
Total	Mean (40)	173	105	95
	S. D.	63	28	21

Discussion:

There is no significant difference between the male and female results in any of the salivas tested. There is a significant difference between the activity of MNS and both parotid and submandibular/sublingual saliva.

"t" Test for Significance:

Male.	MNS and Parotid.	"t" = 5.12 : $p < 0.001$
	MNS and Submandibular/sublingual.	"t" = 5.92 : $p < 0.001$
Female.	MNS and Parotid.	"t" = 3.42 : $p < 0.001$
	MNS and Submandibular/sublingual.	"t" = 4.27 : $p < 0.001$
Total.	MNS and Parotid.	"t" = 4.30 : $p < 0.001$
	MNS and Submandibular/sublingual.	"t" = 5.12 : $p < 0.001$

As the MNS was produced by minimal stimulation, the major contribution (66%) was from the submandibular/sublingual glands (Kerr, 1961).

The/

The submandibular secretions being present in the ratio of 2:1 relative to parotid might lead one to expect a lower or very similar figure for the streptokinase-activated proactivator activity of MNS. The consistently and considerably higher activity of the MNS must therefore be accounted for by other factors.

The major difference between combined salivary secretions, gingival fluid and MNS is the presence in MNS of cells (epithelium, bacteria and leukocytes), and the secretions of the minor salivary glands. The gingival fluid has been shown to contain plasminogen (Gustafsson and Nilsson, 1961) and a later experiment in this work compares the plasminogen levels in MNS of a dentate with an edentate population. A higher level of plasminogen in the dentate population would strongly suggest a significant contribution from the gingival fluid. Dawes and Wood (1973) have reported that the contribution of the minor salivary glands (i.e. labial, lingual, buccal and palatal mucous glands) to whole saliva during rest and when stimulated with sour lemon drops is 8% and 7% respectively. If these glands are responsible for the difference in activity between combined salivary secretions and MNS their secretions must contain very concentrated proactivator. This seems improbable as the secretions are almost entirely mucus, and the literature does not appear to contain any work demonstrating streptokinase-activated proactivator in mucus.

The more probable explanation is that the difference in activity is a result of the action of streptokinase upon the cellular components of MNS. If this is demonstrated to be true, then MNS contains at least two streptokinase-activated proactivator components, one in solution and one cell associated.

Conclusion:

1. MNS is shown to contain streptokinase-activated proactivator(s).
2. All parotid and submandibular/sublingual secretions collected demonstrate/

demonstrate streptokinase-activated proactivator activity. The activity of both these secretions is significantly less than that of MNS.

3. It is suggested that at least two streptokinase-activated proactivator components are present in MNS, one in solution and the other cell associated.

EXPERIMENT 3.

Object: To examine the supernatant of mixed native, parotid and submandibular/sublingual saliva for streptokinase-activated proactivator.

Materials:

1. MNS, minimally stimulated, parotid and submandibular/sublingual salivas were collected (Methodology, p. XXV) from 20 healthy persons, aged 18 - 30 who were taking no drugs and who had no clinical evidence of gingivitis.

2. SBFP. (Methodology, p. IV).

3. Controls. Urokinase, positive. 5 Ploug units/ml. in tris buffer, pH 7.8, 0.15M.
Tris buffer, negative. pH 7.8, 0.15M.
Streptokinase (SK) 2,500 units/ml. in tris buffer, pH 7.8, 0.15M.

Method:

From each salivary sample, 0.5 ml. was removed and mixed 1:1 (v/v) with streptokinase. The mixture was then plated 3 x 0.03 ml. upon SBFP. Control solutions were set up as described in Part II, Experiment 3, Fig. 2.

The remaining MNS was centrifuged at 4154 g. for 30 minutes and from the supernatant 0.5 ml. was removed and the above process with streptokinase repeated. The remaining supernatant was centrifuged at 35,664 g. for 30 minutes and the supernatant processed and plated as above.

The remaining parotid and submandibular/sublingual salivas containing minimal (if any) cellular material were centrifuged at 35,664 g. the/

the supernatant combined with streptokinase and plated as above.

All the plates were then incubated for 19 hours at 37°C.

Results:

These are recorded in Tables 66 - 68 (Appendix 1).

Summary of Results:

Saliva		Saliva/SK	Supernatant of Saliva: 4154 g. after 30 mins.	Supernatant of Saliva; 35664 g. after 30 mins.
MNS (20)	Mean	186	146	122
	S.D.	46	27	18
Parotid (20)	Mean	114		99
	S.D.	23		8
Subm./ subling. (20)	Mean	87		93
	S.D.	42		28

Discussion:

There was a significant difference between the mean activity generated by whole MNS with streptokinase and the mean activity generated by the supernatant of the MNS with streptokinase both at low speed centrifugation ("t" 3.27 x $p < 0.005$) and after high speed centrifugation ("t" 5.64 $p < 0.001$). The difference between the low speed supernatant activity and the high speed supernatant activity was also significant. ("t" 3.23 $p < 0.005$).

There was a significant difference between the mean activity generated by whole parotid saliva with streptokinase and the mean activity generated by submandibular/sublingual saliva with streptokinase ("t" 2.46, $p < 0.025$) However, after high speed centrifugation, there was no significant difference between the activity generated by these salivas. There was a significant/

significant difference between the mean parotid activity before and after centrifugation ("t" 2.68 : $p < 0.01$). If reference is made to the result table 67 (Appendix 1, p. 97) four results, numbers 2, 9, 10 and 11 show a very pronounced drop in activity before and after centrifugation and this suggests that these four were not collected cleanly and that some cellular particles that have come down with centrifugation contaminated the secretion. Without these four results, the difference was only significant at a very low level ("t" 1.5 : $p < 0.2$). With the mean submandibular/sublingual activities there was no significant difference before and after centrifugation.

Comparison of the readings achieved with the high speed supernatant of MNS and those of both parotid and submandibular/sublingual saliva was very interesting. Comparing parotid and MNS supernatant, the difference was significant ("t" 5.09, $p < 0.001$) and comparing submandibular/sublingual saliva with MNS supernatant the difference was also significant ("t" 3.8 : $p < 0.001$). These two results suggest that even when all the cellular components of MNS had been removed, the activity was still greater than that produced by either parotid or submandibular/sublingual salivas alone. It would appear then that some streptokinase-activated activity was present in the supernatant of MNS that was not present in either parotid or submandibular/sublingual saliva. There are four possible sources.

1. Gingival fluid. Although every care was taken to ensure that all the subjects had no clinical evidence of gingivitis, some gingival fluid may have been present in some instances. The gingival fluid is known to contain plasminogen (Gustafsson and Nilsson, 1961).
2. The secretions of the minor salivary glands. With the exception of the serous glands of von Ebner, these are mucus secreting glands which provide only 7 - 8% of the total volume of MNS (Dawes and Wood, 1973) and therefore are unlikely to provide sufficient proactivator to raise the level in the supernatant/

natant significantly.

3. Diffusion of plasminogen through the mucous membrane of the mouth. This would be extremely difficult to establish and was unlikely to have been a major source because the quantity of parotid and submandibular saliva in even minimally stimulated MNS would have so greatly diluted it.
4. Plasminogen may be adsorbed from saliva on to the surface of buccal epithelial cells or even produced by the epithelial cells and when MNS is collected and centrifuged, some of this plasminogen may pass into the solution. Epithelial cells are the most likely 'additional' source and further evidence of such cell associated activity is given in Chapter 3.

Conclusions:

The results (confirming those of Experiment 2) suggest a cell associated proactivator in MNS in addition to one in solution.

CHAPTER 2

EXPERIMENT 1.

Object: To investigate the presence of plasminogen in mixed native, parotid and submandibular/sublingual saliva using two dimensional immunodiffusion.

Two dimensional double diffusion.

Bechhold (1905) first observed immunoprecipitates in a gel medium but the potential for serologists was not appreciated. Well standardised techniques for immunodiffusion were not established until Oudin (1946) gave his first report on the principles and application of the simple diffusion technique in tubes and Ouchterlony (1948) introduced double diffusion. Given a known antiserum, double diffusion allows the direct identification of the corresponding antigen in an unknown mixture.

Principle.

Antigen and antiserum are placed in separate wells cut out of agar in a Petri dish (see Methodology, p. VII). Concentration gradients of both of the reactants are established in such a way that somewhere in the medium, antigen and antibody are present in 'Optimal Ratio' (OR) the concentration at which immunoprecipitation takes place in the shortest time. The precipitation line thus formed will then extend, the rate of extension being inversely proportional to the square root of the time of diffusion.

Comment.

1. pH. 6.5 - 8.2. Outside this range, immunoprecipitation may be retarded and a non-specific precipitation may be induced (Ouchterlony and Nilsson, 1973).

2./

2. The Optimal Ratio. At an excess of the antigen or antibody, the reaction time is increased and the formation of the precipitate is partially or totally inhibited. Where there is an excess of one of the reactants, there may be a reverse of antigen-antibody binding and newly formed aggregates are more sensitive to this dissolving effect than well established precipitates. The double diffusion technique overcomes this problem by allowing the reactants to find their own Optimum Ratio within the medium.

Experiment 1;

Part 1. Sensitivity

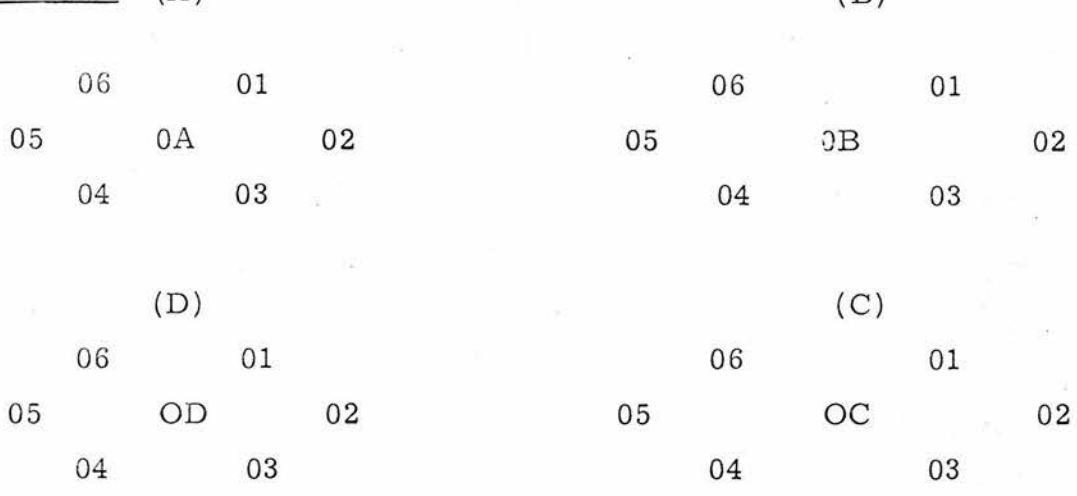
Materials:

1. Two agarose plates were prepared (Methodology, p. VII) and four sets of wells were cut into each plate and labelled A, B, C, and D commencing in the top left corner and proceeding clockwise.
2. Plasminogen. Solution prepared from 'Plasminogen Kabi', a lyophilized powder prepared by affinity chromatography. Supplied in vials each containing human plasminogen equivalent to 25 C.U. (caseinolytic units) and having a purity grade of about 15 CU/mg. protein.
3. Anti-plasminogen serum. Prepared from rabbits by Berhringwerke Laboratories, Germany and supplied in 1 ml. vials.

Method:

A 2.5 CU/ml. solution of plasminogen in tris buffer was prepared and deemed 100%. Serial dilutions in tris were made to 10%. The wells were filled, 0.01 ml., according to the scheme illustrated in Diag. 3.

Diag. 3.



A. Antiplasminogen	B. Antiplasminogen	C. Antiplasminogen
1. Plasminogen 100	1. Plasminogen 40	1. Plasminogen 40
2. 90	2. 30	2. 30
3. 80	3. 20	3. 20
4. 70	4. 10	4. 10
5. 60	5. Tris buffer	5. Tris buffer
6. 50	6. Empty	6. Empty

D. Antiplasminogen
1. Plasminogen 100
2. 90
3. 80
4. 70
5. 60
6. 50

Undiluted anti-plasminogen, 0.01 ml., was placed in the centre well of each set.

After 30 minutes, a further 0.005 ml. of the appropriate solution was added to every well on the plate.

The plates were now placed in a damp chamber at room temperature. The plates were examined twice a day and it was found that no discernable/

cernable extension of the precipitin lines took place after 3 days.

Results:

These are illustrated in Figs. 45 and 46.
In all four tests, the lowest concentration at which a precipitin line was detectable was 30%.
The trial was repeated and the same result was achieved.

Discussion:

At a plasminogen concentration of 100% (2.5CU/ml.) the precipitin line was clear as the aggregates were concentrated in a narrow band at the position of O.R. The line was about 3 mm. from the centre well. As the dilutions progressed, so the precipitin band formed closer to the weaker reactant: the distance from the centre well was 4 mm. at a concentration of 50% and was 5 mm. at 30%. The aggregates were necessarily much fewer at the weaker O.R. and the band correspondingly fainter. When the concentration of the reactants at the precipitin band exceeds the threshold of visibility, a precipitin band can be seen and if the system is balanced, the line remains stationary and its density increases. If the system is considerably unbalanced, the initial precipitate will gradually grow in the direction of the diffusion of the reactant present in excess. Figs. 45 and 46 indicate that at weaker dilutions of plasminogen, the system became progressively unbalanced and the initial precipitate had grown, shown here by a widening of the band. The widening of the band coincided with a progression towards the threshold value for visible aggregation which here occurred at 30%.

When a trial was conducted in which the antiplasminogen in the centre well was doubly diluted to concentrations of 50% and 25%, the precipitin bands were all fainter and the last visible band occurred again in relation to a plasminogen concentration of 30%. When a 25% anti-

Fig. 45.

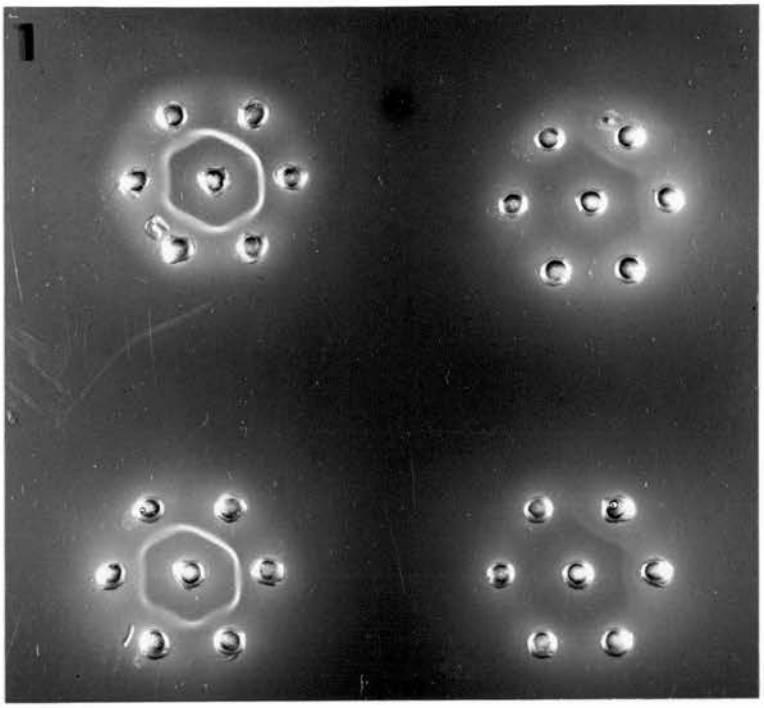
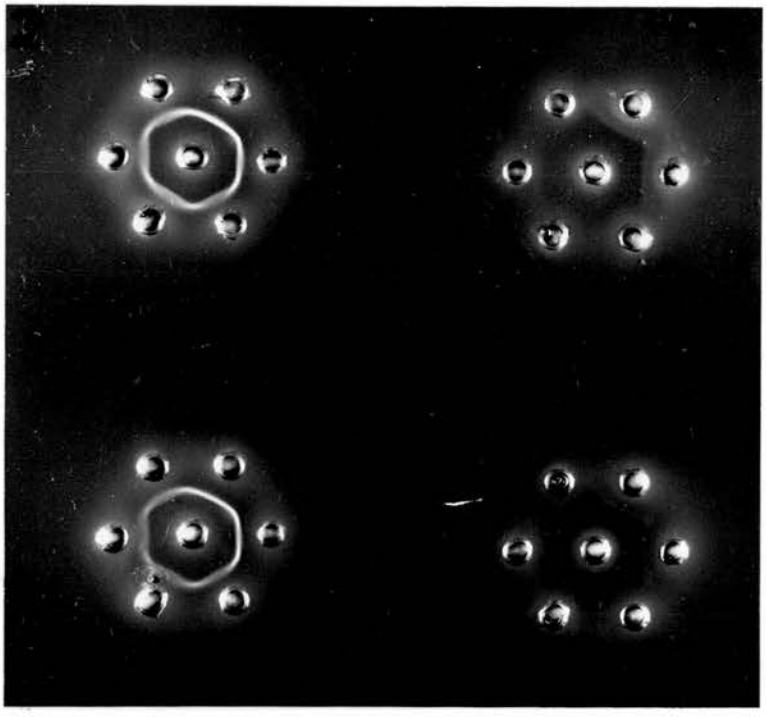


Fig. 46.



Figs. 45 & 46.

Ouchterlony immunodiffusion plates set up as in Diag. 3 and showing a threshold of visibility occurring at 30% of the plasminogen solution.

plasminogen solution was used, the last band (which was extremely faint) was in relation to a plasminogen concentration of 50%. Thus no increase in sensitivity of the system was achieved by lowering the concentration of the anti-plasminogen.

Conclusion:

The lowest concentration of plasminogen that provides a visible precipitin line in this system is 30% of a 2.5 CU/ml. preparation. This is 0.75 CU/ml.

Part 1. Sensitivity (Contd.)

A more sensitive immunodiffusion technique is illustrated in Diag. 4 and is a demonstration of Ouchterlony's Type 1 reaction.

Diagram 4.

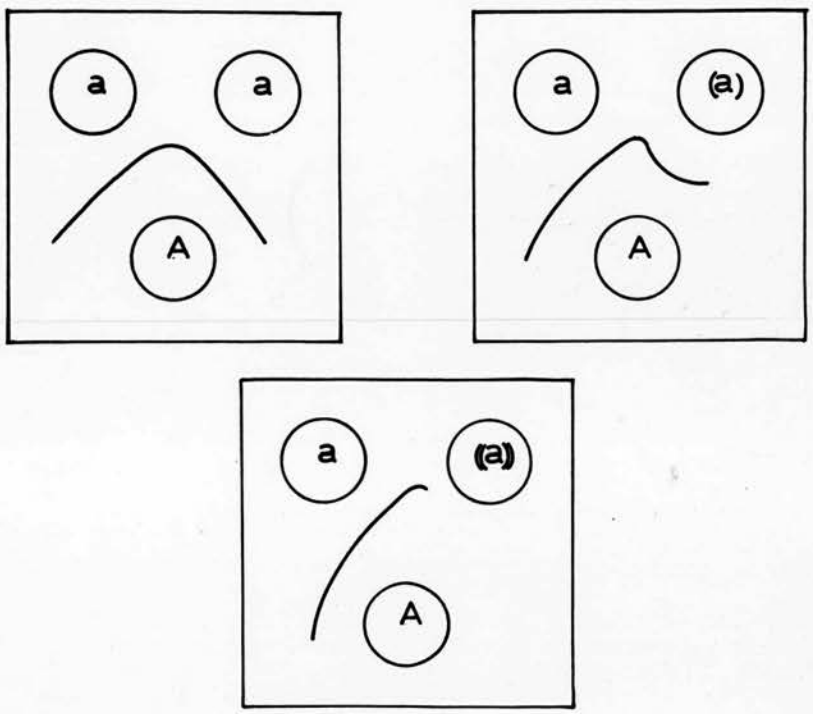
Reaction type 1 and variations when identical antigens at varying concentrations are compared.
In reaction type 1, the precipitin lines from each side deviate and make a complete fusion in the centre area. For balanced systems, this arc formed by the fusing lines is symmetrical. The reaction indicates the presence of identical antigenic determinants in the compared reactants. An asymmetric arc of fusion is seen when the compared reactants are present in distinctly different concentrations in the two wells. When the concentration of one of the compared reactants is below the threshold value for the formation of a visible precipitate, its presence might be revealed by the deviation induced in the pattern on the opposite side. A 'hook' at the end of the precipitin band may be seen curving in towards the antigen of identity.

Ouchterlony. Reaction type 1 and variations.

Materials:/

Diagram 4.

OUCHTERLONY REACTION TYPE 1 AND VARIATIONS WHEN IDENTICAL ANTIGENS AT VARYING CONCENTRATIONS ARE COMPARED



KEY

a = ANTIGEN

A = ANTIBODY (corresponding)

(a) = concentration of antigen less than a

((a)) = concentration of antigen less than (a)

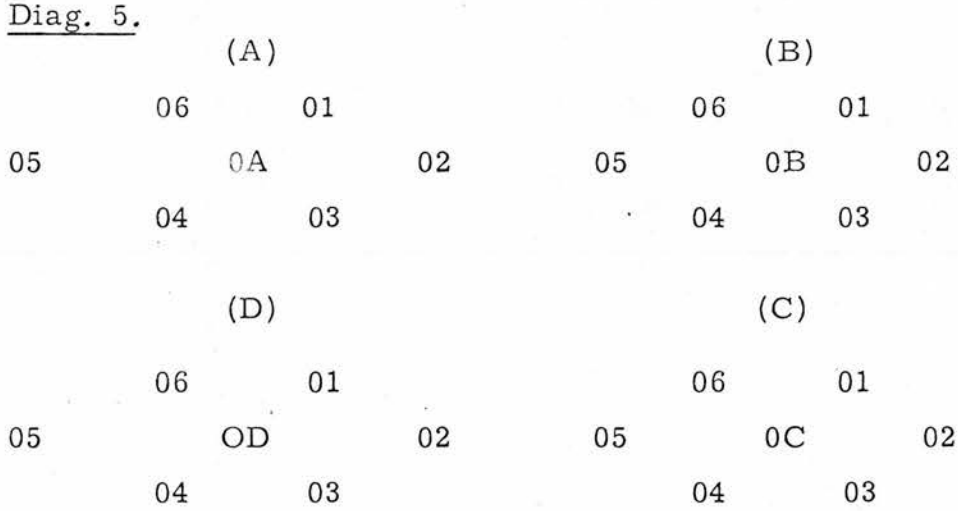
Materials:

Agar plates were prepared as before.
Plasminogen and anti-plasminogen solutions as before.

Method:

A solution of plasminogen was prepared at a concentration of 2.5 CU/ml. (100%) and further dilutions were prepared from 100 - 10% at 10% intervals. The dilutant tris buffer was used as a negative control. The wells were filled as illustrated in Diag. 5.

Diag. 5.



A. Antiplasminogen
1. Plasminogen 100
2. 100
3. Tris buffer
4. Plasminogen 90
5. 100
6. Tris buffer

B. Antiplasminogen
1. Plasminogen 80
2. 100
3. Tris buffer
4. Plasminogen 70
5. 100
6. Tris buffer

C. Antiplasminogen
1. Plasminogen 40
2. 100
3. Tris buffer
4. Plasminogen 30
5. 100
6. Tris buffer

D. Antiplasminogen
1. Plasminogen 60
2. 100
3. Tris buffer
4./

Fig. 47.

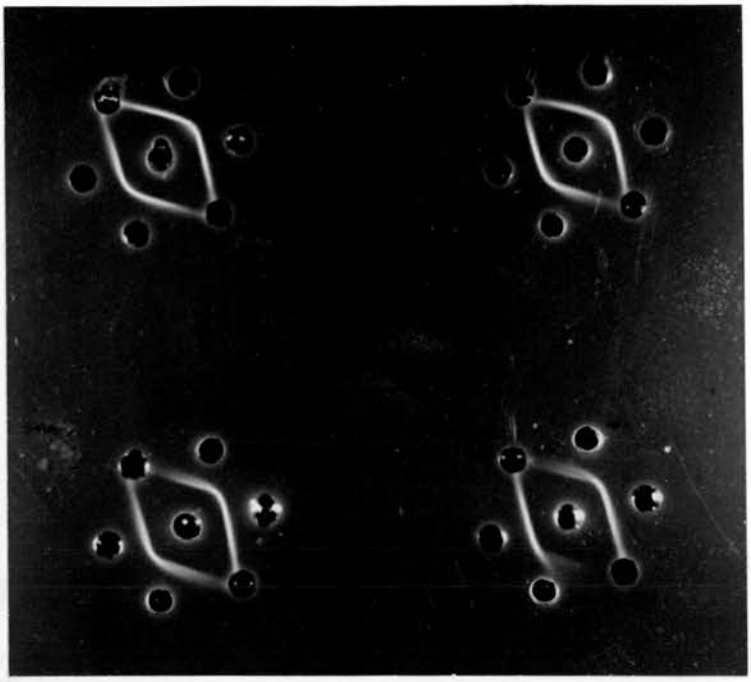
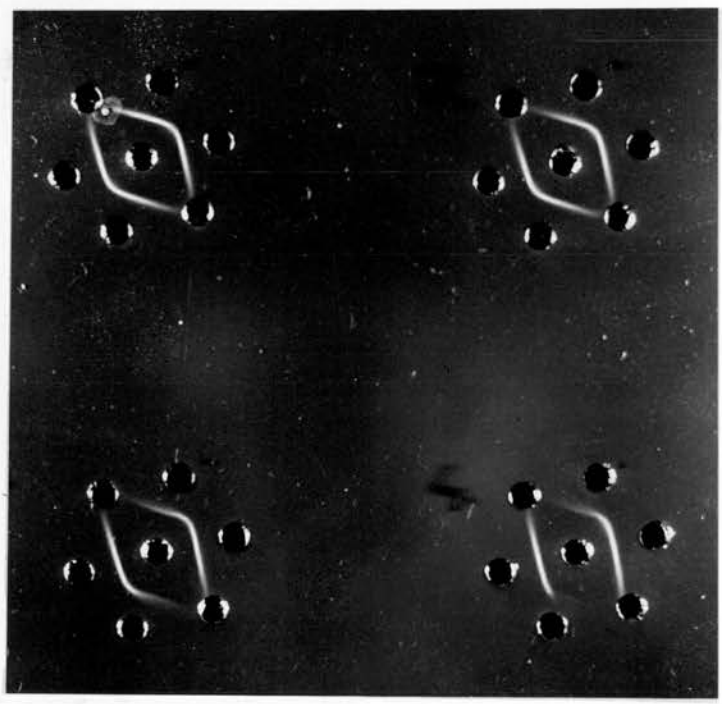


Fig. 48.



Figs. 47 & 48. Ouchterlony immunodiffusion plates set up as in Diag. 5. showing a threshold of visibility at 30% of the plasminogen solution.

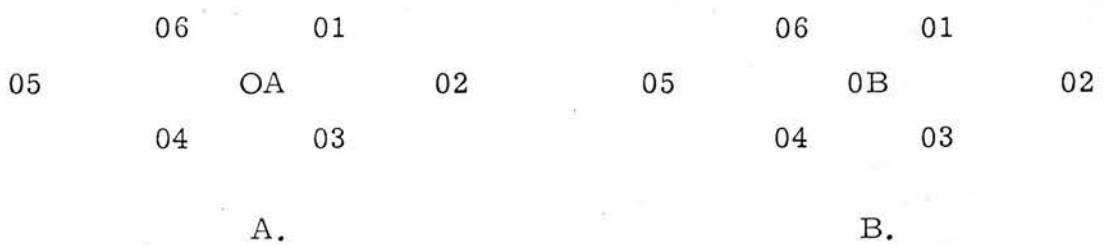
4. Plasminogen 50
5. 100
6. Tris buffer

Results:

Figs. 47 and 48 illustrate the results obtained from two separate trials.
A faint precipitin band was still visible at 30% and the deviation of the adjacent precipitin band formed in relation to a concentration of 100% was clearly seen.

The dilutions were, therefore, extended to 10% plasminogen solutions and dispensed into the plate wells in a similar manner (Diag. 6)

Diag. 6



A. Antiplasminogen
1. Plasminogen 20%
2. Plasminogen 100%
3. Tris buffer
4. Plasminogen 30%
5. Plasminogen 100%
6. Tris buffer.

B. Antiplasminogen
1. Plasminogen 10%
2. Plasminogen 100%
3. Tris buffer
4. Plasminogen 10%
5. Plasminogen 100%
6. Tris buffer.

Result:

A faint precipitin line was visible at 30% (Fig. 49) a slight deviation of the adjacent precipitin line was visible at 20% but no such deviation was/

Fig. 49.

A.

B.

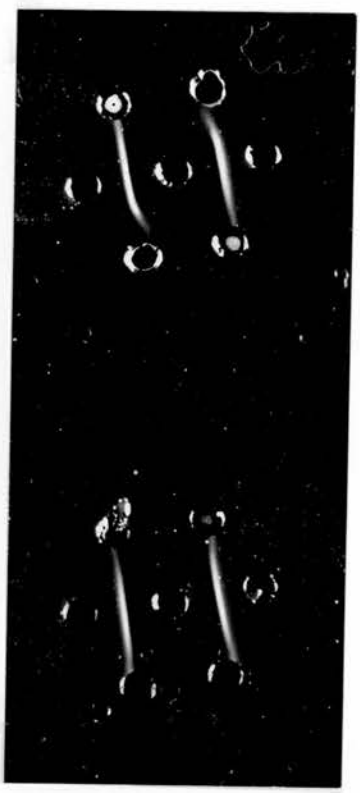


Fig. 49.

Ouchterlony immunodiffusion plate set up as in Diag. 6. showing a slight variation/deviation of the adjacent precipitin line at 20% but not at 10% of the plasminogen solution.

was visible at 10%.

Conclusion:

No accurate quantitative estimation of plasminogen can be made using this technique, but a concentration of plasminogen equivalent to 20% of 2.5 CU/ml. (0.5 CU) may be detected.

Part 1. Sensitivity (continued).

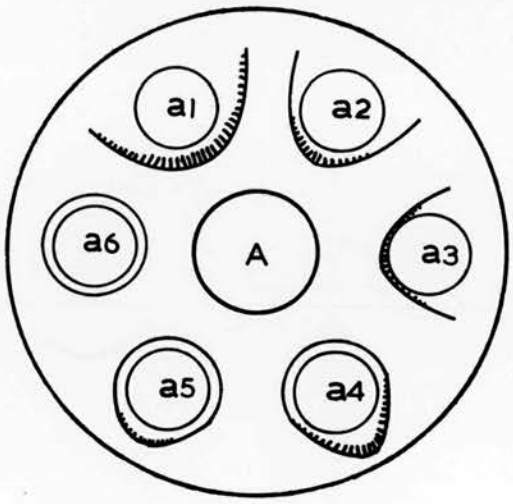
The Gradient Plate Technique. (Feinberg 1957)

Principle:

This technique was devised for quantitative analysis. In a standard agar diffusion plate small circular wells are placed circumferentially around a larger circular well. The antiserum is poured into the centre well and the plate is preincubated for 1 - 3 days, a time estimated for the establishment of an antibody gradient extending beyond the peripheral wells and the plates are incubated for 24 hours. The highest antigen concentration giving a complete ring around the well is noted as the end point of the titration.

Diag. 7.

GRADIENT PLATE TECHNIQUE FOR QUANTITATION.
TWO-DIMENSIONAL DOUBLE DIFFUSION PLATE TECHNIQUE



KEY
a=ANTIGEN in serial dilution from 1 to 6.
A=ANTIBODY

Method:

Two standard agar diffusion plates were prepared and into each were cut two well patterns.

Antiplasminogen (0.66 ml.) was placed in the centre wells. The plate was put into a damp chamber at 4°C and left for 48 hours. At 48 hours a 2.5 CU/ml. solution of plasminogen was prepared in tris buffer and from this solution concentrations of 50, 25, 12.5, 6.25, 3.125 and 1.56% we were prepared by double dilution and were then applied to the wells as indicated in Diag. 8.

Diag. 8

	100	50			100	12.5	
tris buffer	A	25		tris buffer	A	6.25	
	6.25	12.5			1.56	3.125	

Prepared in duplicate (Fig. 50) Prepared in duplicate (Fig. 51)

Result:

The results are shown in Figs. 50 and 51.

The highest antigen concentration giving a complete ring around a well was 6.25% of a 2.5 CU/ml. plasminogen solution. This was equivalent to 0.156 CU/ml. and illustrates the end point of the titration.

Part 1. Conclusion

1. The threshold of visibility using the technique of double diffusion without preincubation is 30% of a 2.5 CU/ml. plasminogen solution (i.e. 0.75 CU/ml.) and using the Ouchterlony TypeI reaction variant the sensitivity is 20% of a 2.5 CU/ml. plasminogen solution (i.e. 0.5 CU/ml.)

2. Using the gradient plate technique, the end point of the titration was determined as 6.25% of a 2.5 CU/ml. solution of plasminogen. That/

That is 0.156 CU/ml.

3. Qualitative evidence, therefore, for the existence of plasminogen in an unknown mixture can be provided by double diffusion provided it is present in a concentration in excess of 0.156 CU/ml.

Fig. 50.

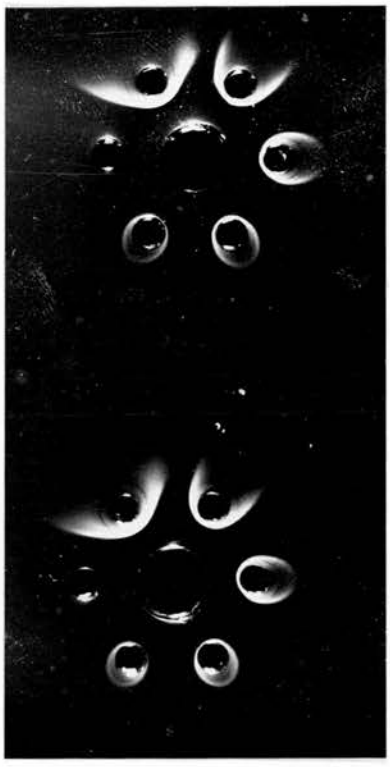


Fig. 51.

Figs. 50 & 51. Ouchterlony immunodiffusion plates set up as in Diag. 8. showing the highest concentration of the plasminogen solution which gave a complete ring around a well was 6.25%.

Experiment 1.

Part 2.

Object: To test for the presence of plasminogen in MNS supernatant of MNS, parotid and submandibular/ sublingual saliva using immunodiffusion techniques.

Materials:

1. Standard agar immunodiffusion plates (Methodology, p. VII)
2. Parotid, submandibular/sublingual and minimally stimulated MNS was collected (Methodology, p. XXV) from 12 healthy adults, aged 18 - 24 who were taking no drugs and who had no clinical evidence of gingivitis. Half of each MNS sample was centrifuged at 4154 g. for 30 minutes to obtain a supernatant.
3. Antiplasminogen serum as described.
4. Human plasminogen (2.5 CU/ml.): positive control.
5. Tris buffer pH 7.8, 0.15M.

Method:

Four sets of wells were cut into each of twelve standard agar plates. Each sample was set up in duplicate and dispensed into appropriate wells after the manner described in diagram 9. The volume dispensed was 0.015 ml. in each well.

Diag. 9.

Key: M Mixed Native Saliva (or parotid or submandibular/ sublingual saliva./

sublingual saliva.

P Plasminogen. Positive control.

T Tris buffer. Negative control.

A Anti-plasminogen.

The prepared plates were placed in a damp chamber and left at room temperature for 3 days.

Results:

A. MNS. No precipitin lines were visible in relation to any of the twelve tested.

B. MNS supernatant. No precipitin lines.

C. Parotid saliva. No precipitin lines.

D. Submandibular/sublingual saliva. No precipitin lines.

E. Positive control. All 48 sets of positive controls displayed clear precipitin lines. No precipitin line deviated towards a test sample well.

As the results were negative, the plates were left a further 3 days in damp chamber but no precipitin lines appeared.

Conclusions:

1. No plasminogen is present in MNS, supernatant of MNS, parotid or submandibular/sublingual saliva.

OR

2. Plasminogen is present in the above substances but is present at a concentration below 0.5 CU/ml., the concentration at which some deviation of the precipitin line of the positive control should occur.

OR

3. The average pore diameter of a 2% agar gel has been estimated at/

at 3 microns. (Ouchterlony and Nilsson, 1973). It is, therefore, possible that the cellular and mucoid components of saliva may impede the diffusion of plasminogen.

OR

4. A combination of 2 and 3.

Procedure 2.

The experiment was repeated using pre-incubated gradient plates in the manner earlier described.
No positive results with the test solutions.

Conclusions:

1. No plasminogen is present in MNS, supernatant of MNS, parotid or submandibular/sublingual saliva.

OR

2. Plasminogen is present in the above substances but is present at at a concentration below 0.156 CU/ml.

OR

3. Diffusion impeded, as above.

OR

4. A combination of 2. and 3.

Experiment 1.

Part 3.

Object: To test for the presence of plasminogen in concentrated mixed native, parotid and submandibular/sublingual saliva and in the concentrated supernatant of MNS.

Preliminary experiments were carried out to compare the effectiveness of various concentrating techniques on a solution of plasminogen (0.3125 CU/ml.) and also MNS.

A. Dialysis tubing:

The test solutions were separately enclosed in dialysis (Visking) tubing and concentrated either by evaporation or by osmotic pressure difference by immersing the tubes in Polyethylene Glycol (P.E.G.) Concentration was estimated by weight difference before and after these procedures. Various combinations of time and temperature were experimented with and it was found that evaporation was very slow and the MNS became a thick sludge which was difficult to handle with both methods. The plasminogen test solution concentrated effectively but with considerable loss of detectable plasminogen as measured upon a Feinberg gradient plate. A volume reduction of 10x only showed a 3 x concentration of plasminogen. With P.E.G. the concentrating process was swift, cheap, and capable of concentrating large volumes of saliva and, therefore, may be useful where only qualitative information is required.

B. Lyphogel: (Polyacrylamide Gel, produced by Gelman Instrument Company and distributed by Hawksley & Sons Ltd.)
Each test solution was placed in a test tube and the granules of Lyphogel were added, sufficient to bring about a concentration of ten times. The concentrating took 5 hours at 4^{o}C. As before, the MNS/

MNS concentrate was unsuitable for pipetting, but the plasminogen test solution was examined as in A and found to have concentrated only two times. Whether plasminogen is partly absorbed or partially inactivated cannot readily be determined, but in either case the method cannot be used for concentrating saliva in order to examine for the presence of plasminogen.

C. Minicon, B15: (Amicon Corp., Lexington, Mass., U.S.A.)

The 'Minicon' B15 is designed to concentrate 5 ml. of test solution to a maximum of 100 times. It is calibrated such that concentrates of 5x, 10x, 25 x, 50x and 100x can be prepared.

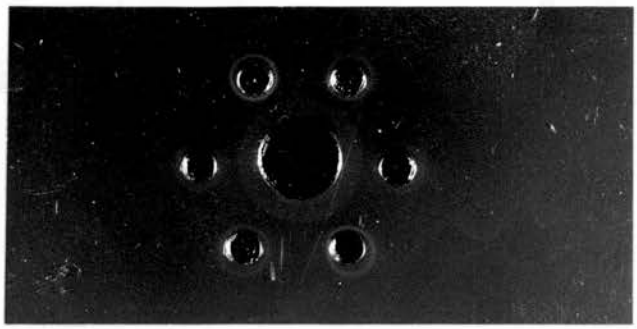
After placing 5 mls. of each test solution in separate channels, the Minicon was stored at 4°C and regularly examined. The plasminogen was concentrated 25x in about one and a half hours but the MNS took about two and a quarter hours to reach this concentration. Of the concentrating techniques described, this was the only one in which it was possible to remove concentrated saliva in a form capable of being pipetted and this was because the Minicon channels permitted the MNS cellular components to settle into a button. The substance removed from the MNS channel was supernatant containing small cellular particles. The MNS concentrate and the plasminogen concentrate were set up in gradient diffusion plates.

Results:

1. MNS. Distinct but faint precipitin line surrounding the well of maximum concentration (Fig. 52). The line corresponded approximately to that produced in the sensitivity experiment by a known plasminogen concentration of 0.156 CU/ml. .

2. Plasminogen. The precipitin line of maximum concentration was very similar to, but perhaps a little weaker than, the positive control. The positive control was 2.5 CU/ml. Thus in this test, an attempt to concentrate 25X, a 0.3125 CU/ml. solution/

Fig. 52. First evidence of any plasminogen in MNS. Distinct but very faint line surrounding the wells of maximum concentration.



Key:

o1 o2

o6 A o3

o5 o4

1. MNS x 25.
2. MNS x 12.5.
3. MNS x 6.25.
4. MNS x 25.
5. MNS x 12.5.
6. MNS x 6.25.

A Antiplasminogen.

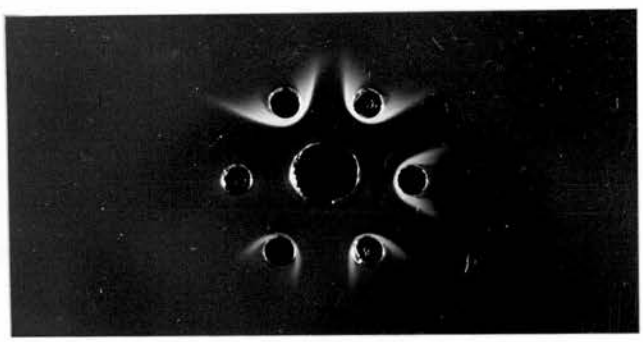
Fig. 53. Concentration control. Test plasminogen solution was 0.3125 CU/ml. concentrated 25x.

Key:

o1 o2

o6 A o3

o5 o4



1. Pl. 2.5 CU/ml.
2. TPl. x 25.
3. TPl. x 12.5.
4. TPl. x 6.25.
5. TPl. x 3.125.
6. Tris buffer.

A Antiplasminogen.

Pl. Plasminogen solution. 2.5 CU/ml.

TPl. Test plasminogen solution (0.3125 CU/ml.) concentrated 25 x. i.e. 7.8 CU/ml.

solution resulted in an actual concentration of about 2.5 CU/ml. or 0.3125 x 8. There was then a considerable loss of plasminogen (Fig. 53).

The threshold of visibility for plasminogen was shown to be about 0.156 CU/ml. in this system. If the effective concentration as suggested by the plasminogen test solution is about 8x then the concentration of plasminogen in MNS, before concentrating, would be approximately 0.156/8. That is 0.0195 or about 0.02 CU/ml. The average pore diameter of 2% agar gel has been estimated at about 3 microns (Weir, 1973) and, therefore, there may have been some inhibition of immunodiffusion due to the many particles in suspension when the MNS was placed in the wells.

Conclusion:

1. Using the Minicon B15 to concentrate a test plasminogen solution, an 8 fold concentration was achieved instead of the calculated 25x.
2. This figure was only approximate as plates would vary in sensitivity and the methods of comparison against positive controls were crude.
3. A precipitin line was formed in relation to concentrated MNS.
4. This precipitin line was distinct but faint and close to the threshold of visibility. The threshold of visibility was about 0.156 CU/ml. Assuming from the control that the concentration of plasminogen in the MNS was also about 8 x, then the estimated concentration of plasminogen in MNS would be in the order of 0.02 CU/ml.
5. The actual level of plasminogen would be higher as diffusion may have been impeded by cellular fragments in the MNS.

Conclusion:

Preliminary/

Preliminary Experiments A - C.

1. Qualitative evidence for the presence of plasminogen in MNS has been presented.
2. No accurate quantitative estimation of plasminogen in MNS was made by this technique as the amount present was very small and the amount lost in concentrating could not be assessed accurately.
3. For the concentration of small quantities of saliva, the Minicon B15 technique was the preferred method whilst dialysis with P. E. G. appeared to be effective in concentrating quickly large quantities of saliva, but the degree of concentration could only be crudely assessed.

Preliminary Experiments (Contd.)

The techniques for concentrating plasminogen were all repeated twice in order that each process would be assessed three times.

On the last occasion the gradients were prepared in the same plate in order that experimental error due to difference in plate sensitivity might be minimised, and also that the results might be photographed under the same conditions, thus enabling a visual comparison of the effectiveness of each method.

The result is described below and illustrated in Fig. 54.

Results:

Experiment A1. Dialysis and evaporation.
Evidence of plasminogen in well 2 and 3 relative to control well 1.
Plasminogen in well 2 should have had a concentration of 3.125 CU/ml. but was evidently much less concentrated than in well 1/

1 in which plasminogen had a concentration 2.5 CU/ml.

Experiment A2. Dialysis and P. E. G.
Evidence of plasminogen in all test wells.
Plasminogen in well 2 should have had a concentration of 3.125 CU/ml. but was evidently less concentrated than in well 1 in which plasminogen has a concentration of 2.5 CU/ml.

Experiment B. Lyphogel.
Evidence of plasminogen in all test wells but as above, evidence of a considerable loss of plasminogen.

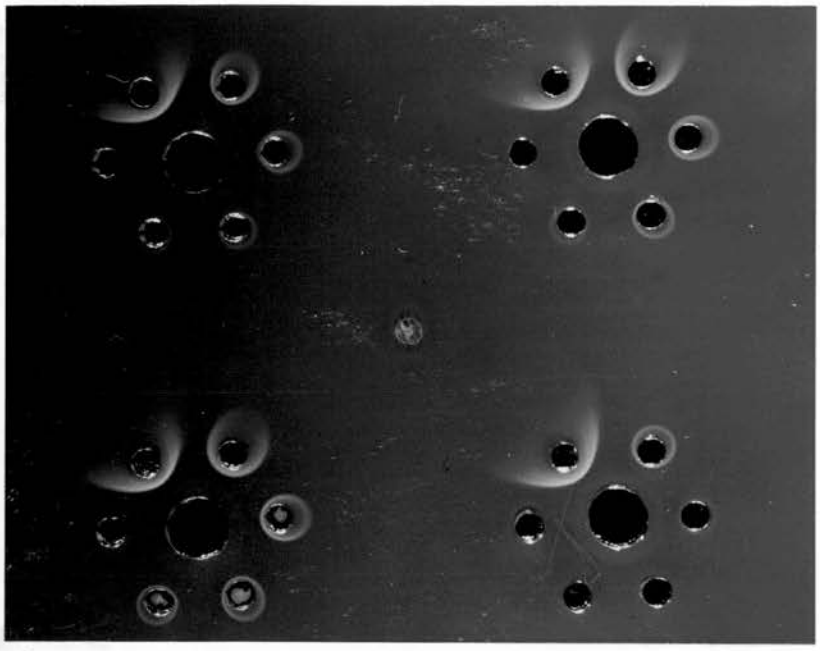
Experiment C. Minicon, B15.
Evidence of plasminogen in all test wells.
In well 2, the concentration of plasminogen was 25 x 0.3125, i.e. 7.8 CU/ml.
It appeared, however, to have a concentration similar to that of the positive control, 2.5 CU/ml. suggesting a concentration of about 8x rather than 25x.

Conclusions:

Confirmed conclusions established for preliminary experiments, A - C.

Fig. 54.

Comparison of the effectiveness of four techniques for concentrating a 0.3125 CU/ml. plasminogen solution assessed by the gradient plate immunodiffusion technique.



01 02		01 02
06 A 03 (B)		06 A 03 (C)
05 04		05 04
01 02		01 02
06 A 03 (A_2)		06 A 03 (A_1)
05 04		05 04

Key. (A_1 & A_2)	(B)	(C)
1. Pl. 2.5 CU/ml.	1. Pl. 2.5 CU/ml.	1. Pl. 2.5 CU/ml.
2. TPl. x 10	2. TPl. x 10	2. TPl. x 25
3. TPl. x 5	3. TPl. x 5	3. TPl. x 12.5
4. TPl. x 2.5	4. TPl. x 2.5	4. TPl. x 6.25
5. TPl. x 1.25	5. TPl. x 1.25	5. TPl. x 3.125
6. Tris. buffer.	6. Tris. buffer.	6. Tris. buffer.

A Antiplasminogen.

TPl. Test Plasminogen Solution. 0.3125 CU/ml.

Experiment 1.

Part 4.

Object: To achieve more definitive immunological evidence of plasminogen in MNS.

Materials:

1. Gradient plates. As described.
2. MNS was collected by stimulated flow (Methodology, p. XXVI) from people working in the Laboratory. All subjects were in good health and had no clinical evidence of gingivitis.

Method:

Freshly collected MNS was centrifuged at 4154 g. for 30 minutes at 4^{o}C. The supernatant was removed and 5 ml. run into one channel of a B15 Minicon. Concentration time varied but on average took two hours to concentrate 25 times.

Gradient plates were prepared two days previous to each test and kept at 4^{o}C. This was to minimize the risk of the antiplasminogen degenerating.
Into the peripheral wells were placed : plasminogen 2.5 CU/ml., positive control: tris buffer, negative control: concentrated MNS supernatant. Each solution was set up in duplicate and, therefore, all peripheral wells were filled. Peripheral wells were refilled after 30 minutes and thereafter the plates were left at 4^{o}C for two to three days.

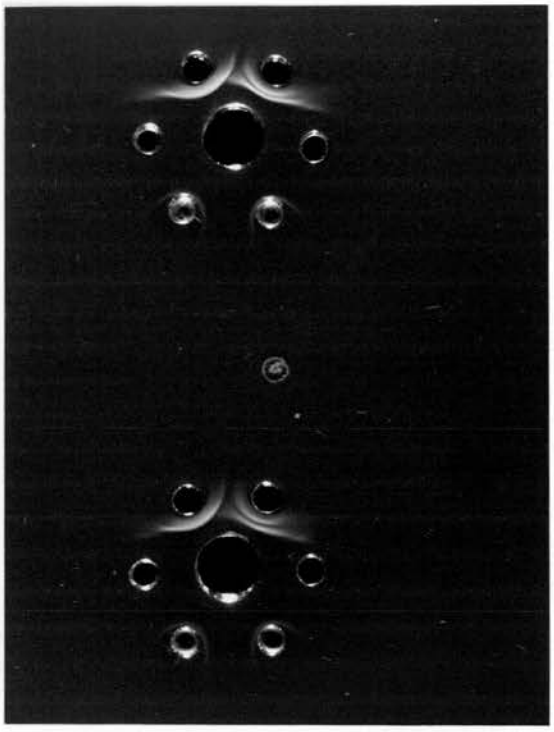
Results:

Precipitin lines to the plasminogen controls were sometimes visible within a few hours but those formed from plasminogen in the MNS were never seen before 24 hours and often not until the second day. All the MNS precipitin lines were faint although consistently present. In/

In an attempt to improve their clarity a "topping up" procedure was tried. As described after 30 minutes, all the peripheral wells were refilled but, in additon, last thing in the evening of the first and first thing in the morning of the second day, the MNS plus one of the plasminogen wells were again refilled from aliquots of the test solutions kept at $4^{o}C$. There was no improvement in the MNS plasminogen precipitin lines and the plasminogen precipitin line from the plasminogen well simply became two precipitin lines.

Fig. 55 illustrates the effect of "topping up" and, in addition, illustrates the faint but very clear plasminogen precipitin lines formed from plasminogen diffusing from MNS supernatant. These experiments, clearly demonstrated the presence of plasminogen in MNS but gave very little reliable information concerning its normal concentration which as suggested before was probably in the order of 0.02 CU/ml. The precipitin line shown here was consistent with that estimate.

Fig. 55.



Key.

01	02	
06	A	03
05	04	

A	Antiplasminogen.
1.	Pl. 2.5 CU/ml.
2.	Pl. 2.5 CU/ml. showing the effect of "topping-up" Cf. well 1.
3.	Tris buffer.
4.	MNS supernatant x 25.
5.	As in 4.
6.	Tris buffer.

The test was set up in duplicate.

Experiment 1. Part 4. (continued)

Object: To examine parotid and submandibular/sublingual saliva for the presence of plasminogen.

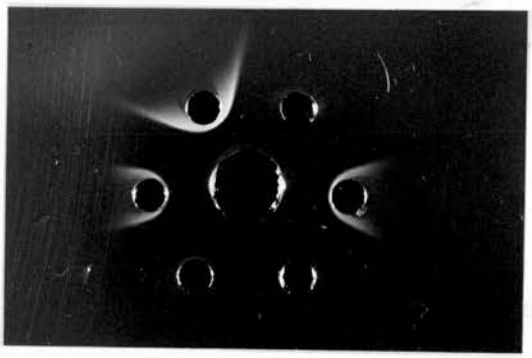
Materials

Stimulated parotid and submandibular/sublingual saliva were collected (Methodology, p XXIX & XXX) from Laboratory staff all of whom were in good health and who had no clinical evidence of gingivitis.

Method

The method was as described for the supernatant of MNS.

Fig. 56. Submandibular/sublingual saliva.



Key.

01 02

06 A 03

05 04

A Antiplasminogen.

1. Pl. 2.5 CU/ml.
2. Tris. buffer.
3. Submandibular/sublingual x 25.
4. Tris. buffer.
5. Tris. buffer.
6. Submandibular/sublingual x 25.

Result

Clear precipitin lines formed in relation to test wells (took two days to appear) and therefore provided evidence of plasminogen in submandibular/sublingual saliva. No accurate quantitative estimation could be made.

Fig. 57. Parotid saliva.

Key.

01 02
06 A 03
05 04

A Antiplasminogen.
1. Pl. 2.5 CU/ml.
2. Tris. buffer.
3. Parotid saliva x 25
4. Tris. buffer.
5. Tris. buffer.
6. Parotid saliva x 25.

Result:

Clear precipitin lines formed in relation to test wells. They took three days to appear.

Discussion:

The precipitin lines in relation to submandibular/sublingual saliva were not always as clear as those illustrated in Fig. 56. However, plasminogen was demonstrated to be present in both submandibular/sublingual, and parotid saliva albeit the lines were faint and the incubation times very long. The inaccuracy of the concentrating method and the length of incubation together with intra plate variation made quantitative estimations by this method unreliable. Parotid plasminogen was not consistently demonstrated after concentrating 25x but parotid saliva took much longer to concentrate than either the submandibular/sublingual or MNS supernatant and there may have been a greater loss of plasminogen. To obtain sufficient parotid saliva for concentration, a minimum of 5 mls. per test was required. In practical terms, this meant collecting from known good secretors who were readily available and so parotid saliva was frequently collected from the same person. Sometimes a precipitation line was obtained and sometimes not, and there appeared to be no rhythm about the occasions when it was 'positive'. A probable explanation is that after the concentrating process, the amount of plasminogen present was only on the threshold of visibility at precipitation.

Conclusion:

Plasminogen has been demonstrated to be consistently present in MNS and submandibular/sublingual saliva, and frequently present in parotid saliva. The inconsistency of the parotid results have been discussed and it is concluded that plasminogen is present in MNS and owes its origin to submandibular/sublingual and parotid saliva with a suggestion that the major contribution may be from the submandibular/sublingual saliva. A more sensitive technique is required before quantitative results can be obtained.

Experiment 2.

Object: To confirm the presence, and to make a quantitative estimation of, plasminogen in human mixed native , parotid and submandibular/sublingual saliva.

Method: Tanned Red Cell Haemagglutination Inhibition Immunoassay (TRCHII).

Principle:

An antigen may be titrated in solution by its capacity to react with antibody and so inhibit the antibody from agglutinating red blood cells coated with the antigen.

Part 1. Introduction.

Part 2. A. Development of TRCHII for the measurement of plasminogen in saliva.
B. Controls.

Part 3. Experiments.

Experiment 2.

Part 1.

Introduction

The limitations of the immunodiffusion techniques have been discussed. From these experiments it was concluded that plasminogen was present in mixed native, parotid and submandibular/sublingual saliva and at a concentration less than 0.1 CU/ml. It was necessary to confirm these findings using a different technique to eliminate the possibility of false positive results and to obtain a more accurate quantitative result.

The sensitivity of the caseinolytic technique is approximately 0.1 CU/ml. (Ludlam and Das, 1971) and is, therefore, unsuitable.

For radioimmunoassay plasminogen must be isotopically labelled, the antigen-antibody mixture must incubate for 72 hours and an expensive scintillation counter is required (Hererlein and Barnhart, 1967: Rabiner et al, 1969). In the absence of the necessary apparatus, an alternative method was required. Haemagglutination inhibition techniques have been used to assay many proteins and are based on the original work of Salk (1944) and Boyden (1951). Merskey et al (1966) and Das (1970) used the method to detect fibrin/fibrinogen degradation products (FDP) and Ludlam and Das (1971) modified the process for plasminogen assay. They were able to demonstrate a sensitivity of 0.013 CU/ml. and a reproducibility that compared favourably with that of the other techniques. The sensitivity that Ludlam and Das achieved is in the correct order of magnitude for an assay of plasminogen in saliva and, in addition, the method is relatively economical. For these reasons, this technique was selected.

Experiment 2.

Part 2A.

Object: To develop the Tanned Red Cell Haemagglutination Inhibition Immunoassay (TRCHII) technique for the measurement of plasminogen in saliva.

For clarity, the complete sequence of events in the modified TRCHII from preparation of reagents to the final calculation of plasminogen in saliva is contained in one sequence in the Methodology, p. X.

The following is a summary of the experiments which led to the modified TRCHII referred to as 'double adsorption'.

1. Experiment.

Object: To perform an antibody titration on plasminogen coated red cells.

Materials:

Microtitre 'V' plate. Cooke Microtiter System, Cooke Engineering Co., Virginia.
Antiserum. Plasminogen antiserum (rabbit). Behringwerke A.G., West Germany.
Diluting Fluid (DF). 2% Bovine serum Albumin. (Methodology, p. X)
Cells. 2.5% suspension of sensitized cells (Methodology, p. XII)

Method:

Two drops of 1/50 antiserum were placed in the first well of a microtitre 'V' plate and one drop of DF placed in the next eleven wells. The antiserum was then serially diluted across the plate. In the second row, one drop of DF was placed in each well to act as a negative control. One drop of cells was added to each well. After gentle shaking the plate was covered, left overnight at 4^{o}C. (Drop volume throughout/

out was 0.025 ml.)

Result:

1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/0	1/2048
+	+	+	+	+	+	+	±	±	−	−	−
−	−	−	−	−	−	−	−	−	−	−	−

Key: (-) No agglutination. (+) Agglutination (±) Intermediate reaction.

The strength of antibody which was the weakest found to agglutinate the cells was 1/64 of 1/50 antiserum, i.e. 1/3200.

Reading the plates. After about 2 to 3 hours, the plate could be read but the result was clearer if a longer time was allowed. The plates in this work were all left overnight and read at about 19 hours.

2. Experiment

Object: To calculate the sensitivity of the assay by performing a haemagglutination inhibition assay using purified plasminogen of known concentrations as a standard.

Method:

Five dilutions of plasminogen solution (Methodology, p. X) were made and two drops of the first dilution added to well A1, two drops of the second to well B1 and so on through to the last dilution in well E1. The solutions were then serially diluted across the wells, one drop of diluting fluid having previously been added to all the other wells. Rows F and G were used as controls. Each well in these two rows contained one drop of diluting fluid.

One drop of antiserum (1/1600) was added to each well except for those in row G, which was used as a negative control, row F being the/

the positive control. The plates were covered and left for 4 hours at 4°C before addition of one drop of 2.5% cells to each well. The plates were incubated overnight at 4°C and the results recorded the next morning.

Results:

Plasminogen dilutions (CU/ml.)

		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
2.5	A	−	−	−	−	−	−	−	−	±	+	+	+
2.0	B	−	−	−	−	−	−	−	−	+	+	+	+
1.5	C	−	−	−	−	−	−	−	+	+	+	+	+
1.0	D	−	−	−	−	−	−	±	+	+	+	+	+
0.5	E	−	−	−	−	±	±	+	+	+	+	+	+
+	F	+	+	+	+	+	+	+	+	+	+	+	+
-	G	−	−	−	−	−	−	−	−	−	−	−	−

The end point was taken as the well with the highest dilution of sample in which no agglutination could be seen.

Sensitivity = concentration of purified plasminogen of standard x titre of standard.

Row A 0.019 Row B 0.016 Row C 0.02 Row D 0.03
Row E 0.06 CU/ml.

Conclusion:

Sensitivity of assay was found to be between 0.016 and 0.06 CU/ml.

As the sensitivity of the assay was in the correct order of magnitude (Ludlam and Das, 1971 achieved a sensitivity of 0.013 CU/ml.) it was hoped that greater consistency of results would be obtained with practice and that an assessment of the effect of saliva upon the prepared cells could be made at once.

3./

3. Experiment.

Object: To assess the effect of saliva on sensitized cells.

Method:

Using a 'V' plate as described, two drops of saliva were placed in well A1. One drop of diluting fluid was placed in the remaining wells of row A and in every well in row B. The sample was serially diluted across the plate and one drop of cells then added to each well. The plate was covered and incubated at 4^{o}C overnight.

Result:

Even very dilute samples of saliva (1/2048) caused the cells to agglutinate due to the presence of some non-specific agglutinin. All the cells in row B (negative control) remained unagglutinated.

Conclusion:

The agglutinin in saliva must be absorbed out or consideration given to the creation of a euglobulin which has been so successful in the estimation of plasminogen in plasma (Ludlam and Das, 1971).

4. Experiment.

Object: To assess the effect of adsorbed saliva upon sensitized cells.

Method:

Details of saliva adsorption are contained in Methodology, p. XIV
At this stage in the development, only a single adsorption was carried out. Following adsorption Experiment 3 above was repeated.

Result:

The/

The agglutinin was diluted out at 1/64 (cf. 1/2048 above) but the suggestion from the immunodiffusion experiments was that if plasminogen was present, it was so at a concentration of less than 0.1 CU/ml. and therefore, a dilution of 1/64 would be too great for this assay system (0.01 - 0.06 CU/ml.) to detect any plasminogen.

All the preceding experiments were repeated several times and it was concluded that :-

a) the sensitivity of the technique must be increased
b) the agglutinin(s) must be removed completely or
c) a combination of both.

5. Experiment.

The sensitivity of the TRCHII can be increased by decreasing the cell concentration.

A series of experiments were performed in which antibody titrations were carried out (as in Experiment 1 above) using varying cell concentrations.

Method:

As in Experiment 1, but in duplicate for each cell concentration. One series being set up in Microtitre 'V' plates and the other in 'U' plates. 'V' and 'U' describe the shape of the vertical section through one of the wells. It was not known at this stage which shape of well would allow the agglutination or inhibited agglutination reactions to be seen most clearly.

Results:

Averaged results from six trials. Strength of antiserum 1/50 (Row 1)

Cell Concentration	Last Positive Reading
2.5 %	1/3200
2.0 %	1/6400
1.5 %/	

1.5 %	1/12800
1.0 %	1/25600
0.75 %	1/25600
0.50%	1/51200
0.25%	Unreadable

Conclusions:

There was a limit below which the cells could not be accurately read (simply due to there being so few) and this was 0.5% with optimal results occurring at 1%. The 'U' plates were not satisfactory as the negative controls did not display clear 'buttons'.

6. Experiment

A series of experiments in which the antibody concentrations was also reduced.

Method:

As above. A short series of six. Antibody concentrations starting at 1/8 of the concentration used above, i.e. starting with 1/400 anti-serum and not 1/50.

Results:

Cell Concentration	Last Positive Reading
2.5 %	1/25600
2.0 %	1/25600
1.5 %1	1/51200
1.0 %	1/51200
0.75 %	1/204800
0.50 %	Unreadable

Conclusion:

Starting with a weaker concentration of antibody the results were easier/

easier to read and less debatable.

7. Experiment.

The sensitivity of the TRCHII was now re-assessed making use of the information obtained above.

Method:

Microtitre 'V' plate. A 1/20 solution of 2.5 CU/ml. plasminogen was prepared and two drops (each 0.025 ml.) were placed in all the wells in row 1(below). One drop of diluting fluid was added to all remaining wells on the plate and the plasminogen solution then serially diluted across the plate as far as row 10. Rows 11 and 12 acted as controls containing at this stage only diluting fluid. Anti-serum was now added, one drop in every well except those in row 12, negative control. The strength of antiserum used was :-

Row A 1/3200, Row B and E 1/6400, Rows C and F 1/12800, Rows D and G 1/51200 and finally in Row H 1/102400. The plate was then covered and left for four hours at 4°C before the addition of one drop of cells to every well. After four hours, one drop of 1% cells was added to every well in Rows A - D and one drop of 0.625% cells to every well in Rows E - H.

The plate was gently tapped, incubated overnight at 4°C, and the results recorded the next morning.

Summary of Results:

The plate.

Antiplasminogen		Plasminogen 1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	1/10240	1/+	1/−
1/3200	A	−	−	±	+	+	+	+	+	+	+	+	−
1/6400	B	−	−	−	±	+	+	+	+	+	+	+	−
1/12800	C	−	−	−	−	±	+	+	+	+	+	+	−
1/51200	D	−	−	−	−	−	±	+	+	+	+	+	−

1/6400	E	−	−	−	±	+	+	+	+	+	+	+	−
1/12800	F	−	−	−	−	±	+	+	+	+	+	+	−
1/51200	G	−	−	−	−	−	+	+	+	+	+	+	−
1/102400	H	−	−	−	−	−	+	+	+	+	+	+	−

(-) No agglutination (+) Agglutination

(±) Intermediate reaction.

Sensitivity = concentration x titre (titre is the value of the last -ve well)

Therefore the sensitivities of the systems A - H are :-

A	2.5/40	0.0625 CU/ml.
B	2.5/80	0.03125 CU/ml.
C	2.5/160	0.0156 CU/ml.
D	2.5/320	0.0078 CU/ml.
E	2.5/80	0.03125 CU/ml.
F	2.5/160	0.0156 CU/ml.
G	2.5/320	0.0078 CU/ml.
H	2.5/320	0.0078 CU/ml.

Conclusion:

The sensitivity of the assay could be increased by using weaker anti-serum and a weaker concentration of cells.

The problem now arose that the system might also have been more sensitive to non-specific agglutinins in saliva.

8. Experiment.

A short series of seven experiments was carried out to assess the effect of adsorbed saliva on different concentrations of cells.
The method was that described in Experiment 4 above. The results were disappointing demonstrating no difference between 2% and 1% cells and no difference from those achieved in Experiment 4. It was apparent/

apparent that although the system had been made more sensitive, it was also more sensitive to non-specific agglutinins in saliva and these were not going to be diluted out and still permit the measurement of plasminogen. The approach had to be changed towards removing the non-specific agglutinins.

A typical result of this experiment is recorded below. The agglutinin is diluted out at 1/64. Even if the system could be kept consistently at a sensitivity of 0.0078 CU/ml. (the greatest sensitivity achieved in Experiment 7) then at least 64 x this amount would require to be present in saliva before it could be detected. This is equivalent to 0.499 CU/ml. and as has been indicated before, plasminogen in saliva is present in concentrations of less than 0.1 CU/ml.

Saliva ⟶

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/ -ve
A	+	+	+	+	+/-	-	-	-	-	-	-	-
B	+	+	+	+	+	+/-	-	-	-	-	-	-

A . Sample of saliva + 2% cells.

B . Sample of saliva + 1% cells.

Discussion:

A highly significant reduction in quantity of non-specific agglutinin in saliva was achieved with a single adsorption. Consideration was given to a second adsorption. If the cells were properly coated with plasminogen, the loss through adsorption of plasminogen in the test sample should be minimal. Ludlam and Das (1971) warn, however, that in estimating plasminogen levels in plasma, the result achieved by haemagglutination inhibition correlates poorly with a caseinolytic assay. Nevertheless, the writer still had to establish qualitatively the presence of plasminogen to confirm the immunodiffusion results rather/

rather than to be able to accurately estimate individual variation, and the method of adsorption was now practiced. Furthermore, providing an estimation of the loss of plasminogen through adsorption could be made a result could still be achieved. The alternative was to attempt to create a euglobulin, precipitate out the plasminogen, redissolve it and then carry out a TRCHII. When this is done with plasma, the plasminogen content of the precipitate is approximately equal to that in plasma (Kline, 1966) and it does not require to be adsorbed since the agglutinin is diluted out before the end point is reached. The method therefore had considerable appeal. The writer digressed from saliva, at this stage, to attempt the euglobulin technique with plasma and compare his results with those obtained by the staff of the coagulation laboratory of the Blood Transfusion Service. At first, poor correlations were obtained, but gradually the technique improved until results were achieved in the same order of magnitude as those results obtained by experienced operators. A decision had to be made at this stage because although the euglobulin technique was improving, there was still the development of a salivary euglobulin to be undergone and materials were expensive and the experiments very time consuming. There was also the worry that even if a result was obtained, it would still have to be evaluated against another technique and thus finally the decision was made to return to adsorption, attempt a double adsorption of saliva and control it as carefully as possible. If this proved successful, then in the certain knowledge that plasminogen was present in saliva and with a clearer indication of the amount present, then would be the occasion to develop a still more accurate quantitative system. The writer considered, at this stage, that the objective of the work was to investigate the fibrinolytic system in MNS in its entirety and to establish knowledge of its component parts and, where possible, their relative contributions. Detailed physiological analysis with the accent upon quantitation rather than qualification is important but subsequent work.

9./

9. Experiment.

Double adsorption.

Full experimental details are contained in Methodology, p. XIV

Summary:

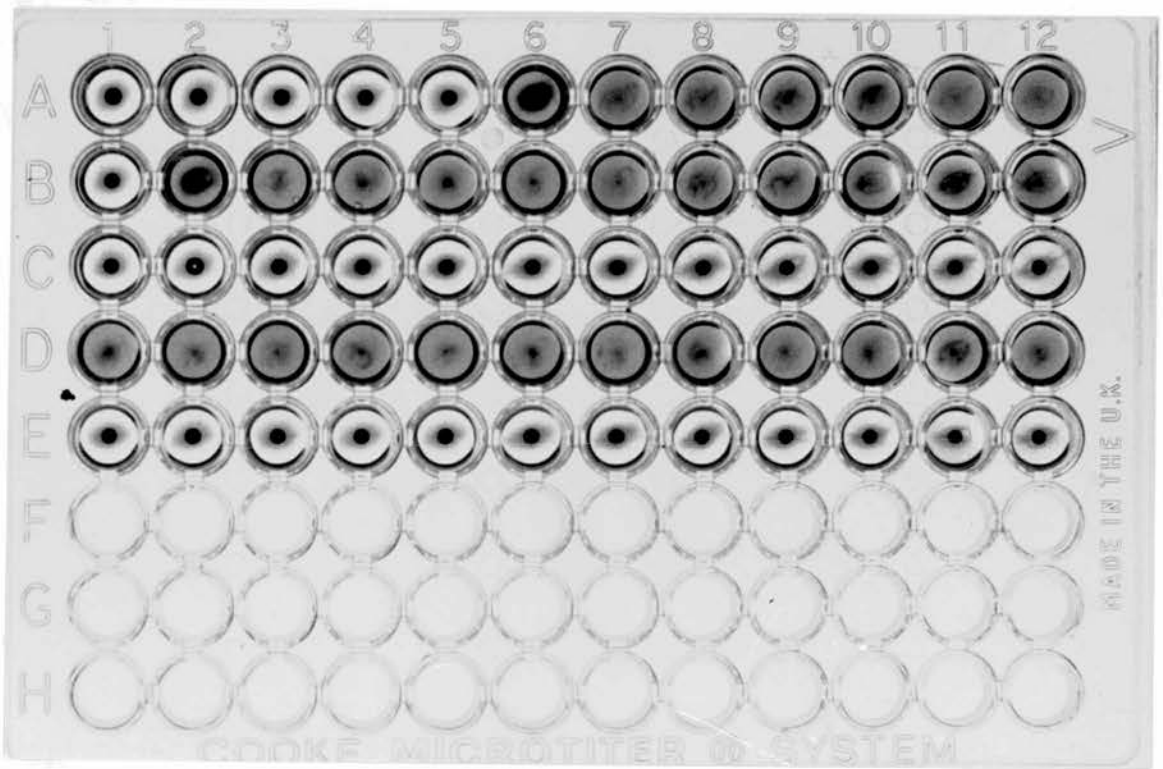
A small sample of MNS (about 1.5 ml.) was collected by minimal stimulation from a subject working in the Laboratory. It was centrifuged at 4154 g. for 30 minutes at 4°C and the supernatant was then removed. Diluting fluid was removed from a 2.5% aliquot of cells and to the cells was added 0.125 ml. of salivary supernatant. The saliva and cells were gently mixed, covered and left at room temperature for one hour. During this time, they were periodically, gently mixed. After one hour, the cells and saliva were transferred to a small centrifuge tube and spun down at 4154 g. for ten minutes. While this was in progress, diluting fluid was carefully removed from a second aliquot of cells. After centrifugation, the saliva supernatant was carefully removed and added to the second aliquot of cells with which it was thoroughly mixed. As before, the saliva and cells were covered and left at room temperature with occasional further mixing. After an hour, the mixture was centrifuged as before and the final supernatant then removed was ready for assay, having been adsorbed twice.

A microtitre plate was prepared and the result is recorded below and illustrated in Fig. 58.

	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	1/2048
A	−	−	−	−	−	±	+	+	+	+	+	+
B	−	±	+	+	+	+	+	+	+	+	+	+
C	−	−	−	−	−	−	−	−	−	−	−	−
D	+	+	+	+	+	+	+	+	+	+	+	+
E	−	−	−	−	−	−	−	−	−	−	−	−
F												
G												
H												

Key/

Fig. 58.



Well B1 no agglutination , a clear button of cells was present in the bottom of the well. This indicates that the antiplasminogen must have been neutralized by plasminogen and that must have been present in the test sample.

Row C shows that saliva that has been doubly adsorbed no longer has an agglutinating effect on the sensitized cells.

Key:

Wells

A1	Plasminogen 1/20
B1	Doubly adsorbed saliva.
C1	Doubly adsorbed saliva negative control.
D1	Positive control.
E1	Negative control.

Reagents

Antiplasminogen 1/3200

Plasminogen 100%: 2.5 CU/ml.

Cells: 2.5%

Result

(+)	Agglutination.
(-)	No agglutination.
(±)	Intermediate reaction.

Calculation

Sensitivity = Standard x titre

$$\frac{2.5}{20} \times \frac{1}{16} = 0.0078 \text{ CU/ml.}$$

Concentration of plasminogen in saliva is sensitivity x 1/titre

$$= 0.0078 \times 1$$

$$= 0.0078 \text{ CU/ml.}$$

On repeating this experiment with the same person's saliva, a result of 0.016 CU/ml. was obtained.

It was noticed at once how very sensitive the system was compared to previous occasions using 2.5% cells and 1/3200 antiplasminogen. At the time, the writer had no explanation other than that with practice, the preparation of cells and the conducting of the experiment had improved. Retrospectively, having prepared many batches of cells, it is now known that despite taking great care, one batch of cells/

cells varies often quite considerably from another in respect of sensitivity and that the sensitivity appears to improve with time. If a batch of sensitized cells is divided up into 2.5 ml. aliquots in diluting fluid, they should not be used for at least four days. In later work, this became a week. The use of fresh diluting fluid at every step was essential. Complete failure to obtain any results with one whole batch of cells was eventually traced to contaminated diluting fluid.

With a positive result being obtained with the very first double adsorption, it was essential to repeat the experiment at once. A comparison of MNS from different individuals was performed. Each sample was controlled as above and set up in duplicate.

Results:

Subject	Concentration of Plasminogen: CU/ml.	
	1.	2.
1.	0.016	0.032
2.	0.016	0.016
3.	0.062	0.062
4.	0.062	0.031

As a consequence of this pilot study, it was concluded that sufficient evidence existed to suggest that plasminogen was indeed present in the supernatant of MNS. It was now necessary to evaluate and control the method.

Experiment 2.

Part 2B.

Controls:

The following experiments were carried out in order to evaluate the method of double adsorption.

1. Specificity.
2. Inhibition of the agglutination reaction, (1) and (2).
3. To carry out a TRCHII on known concentrations of purified plasminogen and compare the results obtained following single and double absorption.
4. To test for the reproducibility of results obtained by the TRCHII system following double adsorption and to make an estimate of the amount of plasminogen lost in the process.

Experiment 1.

Specificity.

Ludlam and Das (1971) list the substances they used to test the specificity of the TRCHII.

These substances were used to test the specificity of this system with exception of heparin which was omitted since it would not be present in any assay in this work.

The substances listed below failed to demonstrate any interference with the reaction. They were added to the haemagglutination inhibition system in concentrations similar to those found physiologically or used pharmacologically. They neither inhibited the agglutination reaction at an antiserum concentration of 1/400 nor did they cause agglutination of the sensitized cells in the absence of antiserum. A further test was the incorporation of these substances into a 1/20 and 1/15 concentration of 2.5 CU/ml. plasminogen solution upon which a TRCHII double adsorption was carried out. There was no interference with the assay relative to control plasminogen solutions tested at the same time.

Substance	Concentration
Gamma Globulin	1 g./100 ml.
Albumin	4 g./100 ml.
Trasylol	100 Kallikrein inactivator units per ml.
Epsilon-n-amino-caproic acid	10^{-5}M

These substances did not interfere with haemagglutination inhibition assay in the concentrations stated.

Experiment 2.

Inhibition of the agglutination reaction (1).

Method:

From a 2.5 CU/ml. solution of plasminogen concentrations of 2.0, 1.5 and 1.0 CU/ml. were prepared and thereafter further dilutions by serial dilution as far as a concentration of 0.0156 CU/ml.

Following the placement of one drop of diluting fluid in every well of a 'V' microtitre plate (except row 1 from A - J) two drops of the prepared plasminogen solutions were placed in wells A - J, one concentration in each well, and then serially diluted across the plate. One drop of antiplasminogen was then placed in all wells except those in row L (negative control). After 4 hours at 4°C, one drop of 2.5% cells was added to every well on the plate. The plate was read after incubating overnight at 4°C.

Result:

Row 1.

Plasminogen Concentration		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
2.5 CU/ml.	A	−	−	−	−	−	−	−	−
2.0 CU/ml.	B	−	−	−	−	−	−	−	−
1.5 CU/ml.	C	−	−	−	−	−	−	−	−
1.0 CU/ml.	D	−	−	−	−	−	−	−	±
0.5 CU/ml.	E	−	−	−	−	−	−	±	±
0.25 CU/ml.	F	−	−	−	−	−	±	±	+
0.125 CU/ml.	G	−	−	−	−	±	±	+	+
0.0625 "	H	−	−	−	±	+	+	+	+
0.03125 "	I	−	−	±	+	+	+	+	+
0.0156 "	J	−	±	+	+	+	+	+	+
Positive Control	K	+	+	+	+	+	+	+	+
Negative Control	L	−	−	−	−	−	−	−	−

Antiserum/

Antiserum 1/3200.

Calculation of results. Sensitivity = concentration x titre.

	Plasminogen Concentration (CU/ml.)	Sensitivity
A.	2.5	Not less than 0.019 CU/ml.
B.	2.0	Not less than 0.016 CU/ml.
C.	1.5	Not less than 0.012 CU/ml.
D.	1.0	0.016 CU/ml.
E.	0.5	0.016 CU/ml.
F.	0.25	0.016 CU/ml.
G.	0.125	0.016 CU/ml.
H.	0.0625	0.016 CU/ml.
I.	0.03125	0.016 CU/ml.
J.	0.0156	0.016 CU/ml.

Discussion:

These results are consistent with those described in Experiment 5, Part 2 (A), Experiment 2.

There are two important findings :-

1. The inhibition titre was proportional to the plasminogen concentration.
2. The sensitivity had remained constant.

The experiment was repeated four times and the same result was achieved. Given that the concentration of a test plasminogen solution is equal to the sensitivity x 1/titre, then provided that the inhibition titre is proportional to plasminogen concentration and that the sensitivity of the system remains constant, an accurate result will be obtained.

The system described above tested purified solutions of plasminogen carefully diluted and no adsorption was carried out. However, the constancy/

constancy of the sensitivity of the system is a function of the sensitized cells and the antiserum concentration. Therefore, provided that for every test, the antiserum strength is known and the sensitivity of cells tested by setting up known plasminogen standards a reliable calculation of plasminogen in a test solution can be made.

Inhibition of the agglutination reaction (2)

To determine whether or not the inhibition titre will vary in proportion to the antiserum concentration given a constant concentration of plasminogen in the test solution.

Method:

One drop of diluting fluid was placed in each well of rows A - H (except row 1) and two drops in every well of row L. Drop volume was 0.025 ml. A plasminogen solution of 1 CU/ml. was prepared and two drops placed in each well of row 1 from A - H and well L1. The plasminogen in row 1 was now serially diluted across the plate using the Microtitre mixer. Antiplasminogen serial dilutions were now prepared from 1/50 to 1/6400. Into every well of row A was placed one drop of 1/6400 antiserum, into every well of row B one drop of 1/3200 and similarly as far as row H into which was placed the 1/50 dilution. The plate was gently tapped and then placed at $4^{o}C$ for four hours. After four hours, 0.025 ml. of 2.5% sensitized cells was added to every well in rows A - H and L. The plate was again gently tapped and then placed at $4^{o}C$, left overnight, and read the following morning.

The result was as follows :-

Variation of inhibition titre with antiserum concentration using a constant amount of plasminogen.

Antiserum/

Antiserum Concentration		1	½	¼	⅛	1/16	1/32	1/64	1/128
1/6400	A.	−	−	−	−	−	−	−	−
1/3200	B.	−	−	−	−	−	−	−	±
1/1600	C.	−	−	−	−	−	−	±	+
1/800	D.	−	−	−	−	−	+	+	+
1/400	E.	−	−	−	−	+	+	+	+
1/200	F.	−	−	−	+	+	+	+	+
1/100	G.	−	+	+	+	+	+	+	+
1/50	H.	−	+	+	+	+	+	+	+
	I.								
	J.								
	K.								
-ve Control.	L.	−	−	−	−	−	−	−	−

Plasminogen Concentration in row 1: 1.0 CU/ml.

Conclusion:

Using known solutions of purified plasminogen the degree of haemagglutination inhibition has been shown to be proportional to the caseinolytic activity of the standard plasminogen solution. The inhibition titre varied in direct proportion to the antiserum concentration using a constant amount of plasminogen.

Experiment 3.

Object: To carry out a TRCHII on a known concentration of purified plasminogen and compare the results obtained following single and double absorption.

Materials and Methods:

1. Plasminogen standard. A 0.2 ml. aliquot of plasminogen solution, 2.5 CU/ml. was thawed and 1 1/15 (0.1666 CU/ml.) and 1 1/20 (0.125 CU/ml.) solutions prepared using diluting fluid.

2. From a similar aliquot of plasminogen and also using diluting fluid three test solutions of concentrations 0.025, 0.050 and 0.075 CU/ml. were prepared.

3. Adsorption of test solutions.
   Single adsorption. Diluting fluid was removed from a 2.5 ml. aliquot of cells. To the cells was added 0.125 ml. of one of the test plasminogen solutions. The cells and test solution were now gently mixed and left at room temperature for one hour with occasional mixing. After one hour, the cells and test solution were transferred to a small centrifuge tube and spun down at 4154 g. at 4°C for 10 minutes. The supernatant generated was then removed and stored at 4°C until it was time to prepare the Microtitre plate. This test solution was now regarded as having been adsorbed once.
   The process was repeated for the other two test solutions.
   Double adsorption. The same process as above was carried out upon further samples of the test solutions as far as the generation of the supernatant. The supernatant formed was now added to a second aliquot of cells, gently mixed, covered and left at room temperature for one hour with occasional mixing. After one hour, the cells and test solution were transferred to a small centrifuge tube and spun down at 4154 g. for ten/

ten minutes at 4°C. The supernatant generated was then removed and stored at 4°C. These test solutions were now regarded as having been adsorbed twice.

4. Preparation of the plate.

A. Diluting fluid. Vol. 0.025 ml. One drop in every well of rows A - E, and G - I except in every case well 1. In rows K and L two drops of diluting fluid in every well.

B. Samples. Two drops of the test solutions were placed in row 1 in the following manner.

Well A1, Plasminogen solution standard 0.125 CU/ml.
Well B1, Plasminogen solution standard 0.166 CU/ml.
Well C1, Plasminogen Test Solution 1. 0.025 CU/ml.
Well D1, Plasminogen Test Solution 2. 0.050 CU/ml.
Well E1, Plasminogen Test Solution 3. 0.075 CU/ml.
Into wells G1, H1 and I1 were also placed two drops of Test Solutions 1, 2 and 3 respectively.

C. The plasminogen solutions both standards and Tests were now serially diluted across the plate.
In series 'A' the Test Solutions were singly adsorbed.
In series 'B' the Test Solutions were doubly adsorbed.
In each series, as may be deduced from above, the Test Solutions were set up in duplicate upon each plate.

D. One drop of antiplasminogen 1/3200 was now added to every well in rows A - E, G - I and to row K.

E. The plate was gently tapped and left at 4°C for four hours.

F. After four hours, one drop of cells was added to every well in rows A-E, G - I, and rows K and L.

G. The plate was gently tapped, covered, left at 4°C overnight. It was read the following morning.

The/

The single adsorption series together with the unadsorbed plasminogen standards was performed four times and, therefore, every Test Solution was set up eight times.

The double adsorption series was set up in a similar manner.

Microtitre Plate for the comparison of single and double adsorption.

	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
A.	−	−	−	−	+	+	+	+
B.	−	−	−	−	+	+	+	+
C.	−	+	+	+	+	+	+	+
D.	−	−	+	+	+	+	+	+
E.	−	−	+	+	+	+	+	+
F.								
G.	−	+	+	+	+	+	+	+
H.	−	−	+	+	+	+	+	+
I.	−	−	+	+	+	+	+	+
J.								
K.	+	+	+	+	+	+	+	+
L.	−	−	−	−	−	−	−	−

Well A1. Plasminogen 0.125 CU/ml.

B1. Plasminogen 0.166 CU/ml.

C1. Test Plasminogen:
1. 0.025 CU/ml.

D1. Test Plasminogen :
2. 0.050 CU/ml.

E1. Test Plasminogen :
3. 0.075 CU/ml.

G1. Test Plasminogen :
1. 0.025 CU/ml.

H1. Test Plasminogen :
2. 0.050 CU/ml.

I1. Test Plasminogen :
3. 0.075 CU/ml.

K. Positive Control.

L. Negative Control.

Antiplasminogen 1/3200.

The titre displayed above was that obtained where the Test plasminogen solutions had been singly adsorbed. The plasminogen solutions in rows A and B had not been adsorbed.

For both single and double adsorption, plasminogen standards, unadsorbed/

sorbed, were set up on every plate. Therefore because the strength of every solution on the plate was known, it was possible to determine the effect of single or double adsorption of purified plasminogen solutions in terms of plasminogen loss.

Results:

Sensitivity = Concentration x titre.
Concentration = Sensitivity x 1/titre.

Plasminogen standard: 1. Sensitivity 0.125 x 1/8 0.0156 CU/ml.
2. Sensitivity 0.166 x 1/8 0.0207 CU/ml.

Test Plasminogen Solutions: (average of 8 tests).

Concentration Known Value	Concentration Single Adsorption	Concentration Double Adsorption
1. 0.025 CU/ml.	0.016 - 0.02 CU/ml.	0.016 - 0.02 CU/ml.
2. 0.050 CU/ml.	0.0312 - 0.0414 Cu/ml.	0.016 - 0.04 CU/ml.
3. 0.075 CU/ml.	0.0312 - 0.0414 CU/ml.	0.0312 - 0.04 CU/ml.

Discussion:

The accuracy of the technique was controlled by the sensitivity of the system and the limitations imposed upon it by serial dilution.
This was well illustrated in Test 3.
The sensitivity of the system calculated from the titre obtained with the 0.166 CU/ml. plasminogen standard was 0.0207 CU/ml. This was poor compared to sensitivities achieved previously of 0.0078 CU/ml. In Test 3, as concentration equals sensitivity x 1/titre, a negative at titre 1/2 would give a concentration of 0.04 CU/ml. whilst the next titre, 1/4 would give a concentration of 0.08 CU/ml. which was clearly impossible given the known, prepared, concentration. Thus the best obtainable value, with this sensitivity, was a little over half the actual concentration. Intermediate results were obtained at 1/4 titre in Test 3 on five occasions, three with single adsorption and/

and two with double adsorption. These had to be disregarded when it came to calculating the concentration, but suggested that even a slight improvement in sensitivity would have achieved a much more accurate result. For example, in Test 3 with a sensitivity of 0.0156 CU/ml. (obtained with the 0.125 CU/ml. standard) and a negative reading at titre 1/4 (five intermediates were obtained) then the calculated concentration of plasminogen would have been 0.0156 x 4, i.e. 0.062 CU/ml. or 83% of the known value (cf. 55% actually obtained).

It was not possible to state that there was no loss of plasminogen from the Test Solutions with a second adsorption relative to the first adsorption because of the lack of sensitivity in the system. It was permissible, however, to suggest that plasminogen could still be detected following the second adsorption and the loss that may take place was insufficient to be recorded by the system when the sensitivity was between 0.0156 - 0.0207 CU/ml.

Experiment 4.

Object: To test for the reproducibility of results obtained by the TRCHII system following double adsorption and to make an estimate of the amount of plasminogen lost in the process.

Materials and Method:

1. Plasminogen standards. A 0.2 ml. aliquot of plasminogen solution, 2.5 CU/ml. was thawed and 1/15 (0.166 CU/ml.) and 1/20 (0.125 CU/ml.) solutions prepared using diluting fluid.

2. From a similar aliquot of plasminogen and also using diluting fluid three test solutions of concentrations 0.1, 0.2 and 0.4 CU/ml. were prepared.

3. MNS. 2 ml. of fresh MNS was collected from a member of the Laboratory staff who had no clinical evidence of gingivitis. The MNS was centrifuged at 4154 g., at 4^{o}C for 30 minutes and then the supernatant was removed and the process described in the previous experiment for double adsorption was carried out. The final adsorbed supernatant was stored at 4^{o}C.

4. The test solutions were doubly adsorbed and stored at 4^{o}C.

5. Preparation of the plate.

   A. Diluting fluid: 0.025 ml. One drop was placed in every well of rows A - J except in each case well 1. In rows K and L, two drops of diluting fluid were placed in each well.

   B. Samples. Two drops of the test solutions were placed in row 1 in the following manner.

Well A1.	Plasminogen standard: 0.125 CU/ml.
Well B1.	Plasminogen standard: 0.166 CU/ml.
Wells C1, E1 and G1.	Two drops each 0.025 ml., of the adsorbed MNS supernatant.

Wells/

Wells D1, F1 and H1. Two drops each 0.025 ml. of the adsorbed plasminogen solutions, 0.4, 0.2 and 0.1 CU/ml. respectively.

C. Into all wells in row 1, was placed one drop of adsorbed MNS.

D. Into all wells in row J was placed one drop of adsorbed 0.4 CU/ml. plasminogen solution.

E. Into all the wells in rows A - H and in row K was added one drop of antiplasminogen, 1/3200, after which the plate was gently tapped, covered and placed at 4°C for four hours.

F. After four hours, one drop of cells was added to every well in every row. The plate was gently tapped, left overnight at 4°C and read the following morning.

The plate is diagrammatically represented below :-

	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
A.	−	−	−	+	+	+	+	+
B.	−	−	−	−	+	+	+	+
C.	−	±	+	+	+	+	+	+
D.	−	−	−	−	±	+	+	+
E.	−	±	+	+	+	+	+	+
F.	−	−	−	+	+	+	+	+
G.	−	±	+	+	+	+	+	+
H.	−	−	±	+	+	+	+	+
I.	−	−	−	−	−	−	−	−
J.	−	−	−	−	−	−	−	−
K.	+	+	+	+	+	+	+	+
L.	−	−	−	−	−	−	−	−

Well 1. Plasminogen 0.125 CU/ml.
1. Plasminogen 0.166 CU/ml.
1. Adsorbed MNS.
1. Adsorbed plasminogen, 0.4 CU/ml.
1. Adsorbed MNS.
1. Adsorbed plasminogen, 0.2 CU/ml.
1. Adsorbed MNS.
1. Adsorbed plasminogen, 0.1 CU/ml.

Row I. Adsorbed MNS negative control.

Row J. Negative control, adsorbed plasminogen.

Row K. Positive control, reagent.

Row L. Negative control, reagent.

Key: (-) No agglutination (+) Agglutination (±) Intermediate reaction.
MNS. The same specimen set up in triplicate.
Antiplasminogen, 1/3200.

Results:

Sensitivity. Row A. Sensitivity is Conc. x titre. 0.125 x 1/4 : 0.0312 CU/ml.

Row/

Row B. Sensitivity is Conc. x titre. 0.166 x 1/8: 0.0208 CU/ml.

The average sensitivity was therefore 0.026 CU/ml.

The last negative agglutination for MNS appeared in all three instances in row 1, although an intermediate reaction occurred in each case in row 2. This suggests a level of plasminogen in excess of that indicated by the titre 1, but less than that if the titre were 1/2. However, the criteria for reading the plate includes the requirement to accept the last negative as the titre and therefore the value for the plasminogen concentration of MNS must be taken at titre 1 and was therefore 0.026 CU/ml.

Row D. The last negative occurred at titre 1/8. This was equivalent to 8 x 0.026 CU/ml. (0.208 CU/ml.)
The actual concentration before adsorption was 0.4 CU/ml. The titre gave a value of 52% of the actual value.

Row F. The last negative occurred at titre 1/4. This was equivalent to 4 x 0.026 CU/ml. (0.104 CU/ml.) The actual concentration before adsorption was 0.2 CU/ml. The titre gave a value of 52% of the actual value.

Row H. The last negative occurred at titre 1/2 with an intermediate value at 1/4. The actual concentration before adsorption was 0.1 CU/ml. The titre gave a value of 0.026 x 2 (0.052 CU/ml.) 52% of the actual value.

All negative controls were valid.

This experiment was repeated eight times using varying concentrations of plasminogen. The calculated titres in all cases gave a value of 52% of the actual value.

Discussion:

The/

The stipulation that the last negative reading must be considered the titre for any one sample means that unless there is absolutely no loss of plasminogen at all, then the highest titre obtainable will give a reading approximately 50% of the actual figure since the titres are prepared in serial dilution. Therefore, the above experiment indicates a loss of plasminogen not exceeding 50% of the actual amount present and may disguise a loss of considerably less. It is not, therefore, surprising that the apparent loss in each case is the same.

The technique of double adsorption may very well incur a greater loss than single adsorption, but it is not detectable provided the loss is not greater than 50% of the actual amount present. In no case did such a loss occur. The consequence of this is that a calculation of twice the amount estimated from the titre would represent the upper limit of plasminogen concentration in any given sample. It would, therefore seem more accurate to state a range of values rather than a single figure.

e.g. In the test described above, the plasminogen concentration for the MNS is between 0.026 - 0.052 CU/ml.

This experiment was repeated in principle, but with a minor variation. In place of adsorbed purified plasminogen solutions, adsorbed (doubly) solutions of MNS and purified plasminogen in the ratio 1 : 1 (v/v) were prepared. The actual amount of plasminogen added to the MNS before adsorption would be precisely known. The amount of plasminogen in the MNS would be calculated from control MNS by calculation for the titre of the control MNS. In the light of the last series of experiments, the minimum value for the MNS would be known and, therefore, the minimum value for the mixture could be calculated. A result in the order of 50% of this calculated value would substantiate the preceding experiment and suggest that the presence of MNS did not greatly interfere with the plasminogen added to it.

Preparation of the plate:

The preparation of the plate was as described in the preceding experiment. The plate is diagrammatically represented below :-

	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
A.	−	−	−	±	+	+	+	+
B.	−	−	−	−	±	+	+	+
C.	−	+	+	+	+	+	+	+
D.	−	−	+	+	+	+	+	+
E.	−	+	+	+	+	+	+	+
F.	−	−	±	+	+	+	+	+
G.	−	+ −	+	+	+	+	+	+
H.	−	−	−	+	+	+	+	+
I.	−	−	−	−	−	−	−	−
J.	−	−	−	−	−	−	−	−
K.	+	+	+	+	+	+	+	+
L.	−	−	−	−	−	−	−	−

Well 1. Plasminogen 0.125 CU/ml.

1. Plasminogen 0.166 CU/ml.

1. MNS

1. MNS plus plasminogen (0.1 CU/ml.)

1. MNS

1. MNS plus plasminogen (0.2 CU/ml.)

1. MNS

1. MNS plus plasminogen (0.4 CU/ml.)

Row I. Adsorbed MNS negative control.

Row J. Adsorbed MNS plus plasminogen control.

Row K. Reagent positive control.

Row L. Reagent negative control.

Key:

(-) No agglutination. (+) Agglutination. (±) Intermediate Result.

MNS. The same specimen used in triplicate.

MNS mixed with plasminogen was derived also from the one MNS specimen.

Antiplasminogen 1/3200.

Results:

Sensitivity. Row A. Calculated as before. 0.0312 CU/ml.

Row B. 0.0208 CU/ml.

The average sensitivity was, therefore, 0.026 CU/ml.

MNS/

MNS. Concentration is sensitivity x 1/titre and, therefore, the value for the concentration of plasminogen in MNS was 0.026 CU/ml. (minimum).

Row D. The last negative occurs at 1/2 and, therefore, the concentration of plasminogen was not less than 0.052 CU/ml.
Calculated value. MNS : Plasminogen, 1:1.

$$\frac{0.026}{2} + \frac{0.1}{2} = 0.013 + 0.05 = \underline{0.063.}$$

Estimated concentration was about $\frac{0.052}{0.063} \times 100 = 82\%$ of calculated concentration.

Row F. The last negative occurs at 1/2 and, therefore, the concentration of plasminogen was not less than 0.052 CU/ml.
Calculated value. MNS : Plasminogen, 1:1.

$$\frac{0.026}{2} + \frac{0.2}{2} = \underline{0.113.}$$

Estimated concentration was about $\frac{0.052}{0.113} \times 100 = 46\%$ of calculated concentration.

Row H. The last negative occurs at 1/4 and the concentration of plasminogen was not less than 0.104 CU/ml.
Calculated value. MNS : Plasminogen, 1:1.

$0.013 + 0.2 = \underline{0.213}$ CU/ml.

Estimated concentration was $\frac{0.104}{0.213} \times 100 = 49\%$ of calculated concentration.

All negative controls are valid.
This experiment was also repeated eight times using varying concentrations of plasminogen and different specimens of saliva; MNS and MNS plus plasminogen were each set up in triplicate, as above, on each plate.

Discussion:

The results varied compared to those in the preceding experiment. This was due in part to the fact that the 'calculated' value was an estimation based upon the MNS titre which was indicative of about 50% of the plasminogen concentration in a sample of MNS.

From the series of experiments, the mean estimated concentration of plasminogen derived from the titres and the sensitivity of the plates was 55% of the calculated value. In the light of the preceding series of experiments, this is probably too high. A practical working figure, until such time as a more sensitive mechanism than double adsorption is evolved, is 50%.

Conclusion:

The series of control experiments show that the TRCHII modified by the process of double adsorption can be used qualitatively to affirm the presence of plasminogen in saliva. A quantitative estimation is also possible but an exact figure cannot be given. The figure calculated by this method represents about 50% of the plasminogen present.

Experiment 2.

Part 3(1).

Object: To examine the MNS of an edentate and dentate population for the presence of plasminogen.

Method:

Tanned Red Cell Haemagglutination Inhibition Immunoassay. TRCHII modified by double adsorption.

Materials:

1. As described in Methodology, p. X.
2. Mixed Native Saliva. MNS.

Subjects:

1. Dentate: 25 Males, 25 Females. Aged 18 - 40, who had no clinical evidence of gingivitis.
2. Edentate: 25 Males, 25 Females. Aged 20 - 82.
   Each subject was carefully examined for evidence of palatal stomatitis resulting from chronic denture irritation and any other inflammatory reaction in the mouth. Only those subjects with healthy mucous membranes were accepted.

Collection of Saliva:

Saliva was collected using the method described in Methodology, p. XXV) for the collection of saliva with minimal stimulation. Approximately 0.5 - 1.5 ml. was collected from each subject. Immediately after collection, the saliva sample was centrifuged at 4154 g. at 4^{o}C for 30 minutes. The supernatant was then stored at 4^{o}C until the adsorption process commenced. With most specimens this started as soon as the supernatant was prepared.

Procedure: /

Procedure:

The saliva samples were adsorbed and the Microtitre plate prepared in accordance with the method described in Methodology, p. XVI.

Results:

Concentration of plasminogen in the test sample = Sensitivity of assay x 1/titre of sample.

The sensitivity of every test was determined from the titres obtained with the two control plasminogen standard solutions set up on every plate. The concentration of these standards was known in Caseinolytic Units/ml. (CU/ml.) and therefore it was possible to express the concentration of the test samples in the same units.
Every test was set up in duplicate.

The results are recorded in Tables 69 and 70 (Appendix 1).

Summary of Results:

Dentates:

Subjects	Number	Concentration of Plasminogen CU/ml.
Male	25	0.053 ± 0.02
Female	25	0.049 ± 0.03
Total	50	0.051 ± 0.025

Edentates:

Subjects	Number	Concentration of Plasminogen CU/ml.
Male	25	0.055 ± 0.02
Female	25	0.058 ± 0.03
Total	50	0.056 ± 0.026

All Subjects (100) 0.054 ± 0.025

These results record the calculated amount of plasminogen in a sample of MNS. It has been demonstrated in the control experiments that approximately/

approximately a 50% loss can be expected and this must be borne in mind when interpreting the figures. The numerical value should be understood to mean "not less than x CU/ml."

Analysis and Discussion of Results:

Dentates

94% of samples contained detectable plasminogen.
There was no significant difference between the amount of plasminogen detected in male saliva compared to that detected in female saliva ("t" = 0.54).

Edentates

96% of samples contained detectable plasminogen.
There was no significant difference between the amount of plasminogen detected in male saliva compared to that detected in female saliva. ("t" = 0.408).

Combined total of edentates and dentates.
95% of samples contained detectable plasminogen.
Mean value for plasminogen (100) 0.054 ± 0.025 CU/ml.
There was no significant difference between the amount of plasminogen detected in dentate saliva compared to that detected in edentate saliva. ("t" = 0.969).

As there was no significant difference between the measurable amount of plasminogen present in dentate saliva compared with that in edentate saliva, the contribution of plasminogen from the gingival fluid to the MNS must, in the uninflamed mouth, be also insignificant. Higher plasminogen levels might be detected in the MNS of patients suffering from acute gingivitis where the gingival fluid contribution would be greatly increased.

It has now been established that the gingival fluid contributes an insignificant amount of plasminogen to the MNS in the healthy mouth and, therefore, /

therefore, the most probable source is the secretions of the major salivary glands. If their secretions contain plasminogen in the same order of magnitude as the supernatant of MNS, then the major sources will have been established.

Experiment 2.

Part 3(2).

Object: To examine the parotid and submandibular saliva of a dentate population for the presence of plasminogen.

Materials:

Parotid and submandibular/sublingual saliva were collected (Methodology, p. XXIX) from twelve subjects, aged between 18 - 35, who were in good health, taking no drugs, and who had no clinical evidence of gingivitis. Approximately 0.5 - 1.0 ml. of each saliva was collected from each subject.

Method:

Double adsorption and TRCHII as described in Methodology, p. X.

Results:

These are recorded in Table 71 (Appendix 1).

Summary of results :-

Subjects	Number	Concentration of Plasminogen: CU/ml.	
		Parotid	Submandibular/sublingual
Male and Female	12	0.049 ± 0.02	0.051 ± 0.02

Discussion:

The sample was small but adequately demonstrated in the twelve parotid and submandibular salivas plasminogen to be present in the same order of magnitude as in the MNS. No negative results were obtained. These results confirm the immunodiffusion findings that plasminogen is present in both parotid and submandibular/sublingual saliva and whilst not excluding the possibility of a contribution by diffusion/

diffusion through the mucous membrane, the plasminogen in MNS can adequately be accounted for by that present in the above salivas.

Experiment 3.

Gel Chromatography

Principle:

Gel chromatography is a separation method based on differences in molecular dimensions. The tool for obtaining a separation is the chromatographic bed which consists of minute particles usually packed into a tube. The space between the particles is occupied by a liquid which is made to flow through the bed, and carries the substances to be separated. The separation is in accord with molecular weight. Large molecules will emerge first from the bed while smaller molecules are retarded because the small molecules can pass into the gel particles and lag behind the large molecules that cannot penetrate into the gel.

Object:

1. To fraction the supernatant of MNS and determine the absence or presence of plasminogen in the fractions by observing whether or not lysis of bovine fibrin takes place after combining the fractions with streptokinase and also by direct measurement using TRCHII.
2. To observe whether or not those fractions that appear to contain plasminogen contain substances whose molecular weight is in the same order of magnitude as that of plasminogen.
3. To determine whether or not any of the fractions have any activator activity.

Materials and Methods:

Chromatograph Gel. Sephadex G200. A dextran gel, medium grade, of particle size 40 - 120 micron diameter. Fractionation range 5,000 - 800,000 (Pharmacia Fine Chemicals AB, Box 604 S-751 Uppsala 1, Sweden).

Treatment/

Treatment of Gel. Fines were removed by decantation 10x with saline before packing. This achieves better resolution and a better flow rate.

Elution Medium. AG azide saline. (Aronson-Gronwal azide saline and comprises 25mM tris, 4mM borate, 3mM NaN_3, 1mM EDTA and 0.15 M NaCl at pH 8.6.

Eluting conditions. Flow 20 ml./hour. 15 minute fractions each of 5 ml. at 20°C.

Optical density. The optical density of the fractions was measured at 280 nm on a spectrophotometer in 1 cm. cells.

Standard human fibrin plates (Methodology, p. I).

Standard bovine fibrin plates (Methodology, p. IV).

Reagents and equipment for Tanned Red Cell Haemagglutination Inhibition Immunoassay (TRCHII). See Methodology, p. X.

Streptokinase: 2,500 units/ml.

Urokinase: 5 Ploug units/ml. pH 7.8, 0.15M.

Tris buffer, pH 7.8, 0.15M.

MNS supernatant. MNS was collected in accordance with the method described in Methodology, p. XXVI for the collection of stimulated MNS. The supernatant was prepared by centrifugation at 4154 g. for 30 minutes followed by centrifugation at 14,000 r.p.m., 28,000 g. for one hour.

Subjects. All subjects were aged 18 - 33, in good health, were taking no drugs and had no clinical evidence of gingivitis.

Procedure:

About 110 ml. of MNS supernatant was concentrated in an ultra-filtration cell (Amicon, PM10) to between 8 - 10 ml. to form the test sample./

sample.

The density of the sample was not greater than the eluant in this system and therefore glucose (50 mg.) was added to it. (The glucose is eluted later than the test substances and does not disturb the experiment).

The sample was now applied as follows. The eluant above the surface of the bed was removed until a layer about 1 cm. thick remained. The sample was then layered on manually under the surface of the eluant with a long Pasteur pipette. The sample solution, now denser than the eluant, displaced it, formed an even layer on top of the bed and was then carried into the bed by the flow. When the sample had passed into the bed, the space above the top of the bed was filled with eluant and the column connected to the eluant reservoir. The eluting conditions allowed a flow of 20 ml./hour and the 15 minute fractions (each 5 ml.) were collected automatically. After 20 hours, the fractions were removed and the run stopped. The optical density of each fraction was measured at 280 nm (maximum absorption by amino acids) and the protein profile plotted.

Calculation:

The glucose is detected on elution with a Clinistick and the point (fraction) of elution gives V_s (elution volume of the solvent).
V_o Void volume. This is the volume of the liquid in the interstitial space between the grains in the bed. It is determined by a substance that is not retarded by the bed material by measurement of its elution volume. As it is completely excluded, it gives a very sharp peak after which the protein profile for the sample appears. Thus V_o can be determined by visual inspection of the protein profile of the complete run. Now the volume of liquid, for small samples, that has passed the column between the application of the sample and elution of the maximum concentration of a particular substance is the elution volume of that substance, V_e. In this experiment, the substance being looked for was plasminogen./

plasminogen. Every fraction from a run was stored at 4°C. From each fraction was removed 0.5 ml. of solution which was then mixed 1:1 with streptokinase (2,500 units). This mixture was then plated (3 x 0.03 ml.) upon SBFP and incubated at 37°C for 19 hours. Evidence of lysis was interpreted as being suggestive of the presence of plasminogen. Reference was now made to the protein profile for the saliva from which the fractions were derived and those fractions containing "plasminogen" marked. The most concentrated fraction effectively gives V_e.

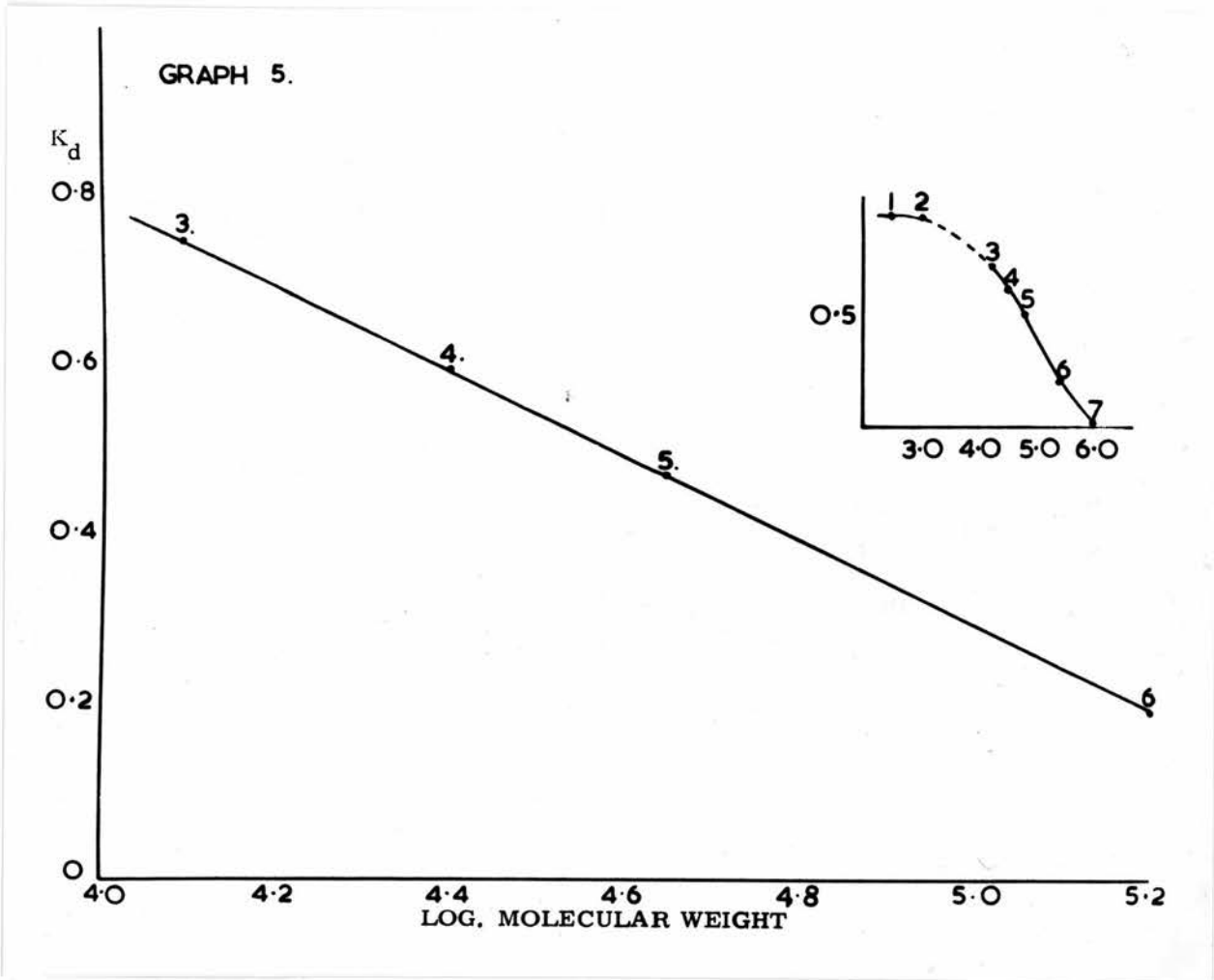
The elution behaviour from Gel columns has been found to be so reproducible that columns do not need frequent recalibration and that for a particular Gel, here G200, a standard reference curve (Graph 5) can be drawn describing the relationship between elution behaviour and molecular weight. It is obtained by applying samples of known molecular weight such as IgG (158,000) Ovalbumin (45,000) and albumin (68,000).

Now if only the liquid imbibed in the gel is assumed to be the stationary phase, then the partition coefficient K_d may be defined as :-

$$K_d = \frac{V_e - V_o}{V_s - V_o}$$

K_d will vary from 0 - 1, from void volume to solvent volume respectively. A relationship exists between K_d and the molecular weight. Thus having obtained V_o, V_s and V_e experimentally, K_d can be calculated with reference to V_e and as the molecular weight is accurately known K_d can be plotted against the log of the molecular weight. Given three known molecular weights, preferably more, the curve can be plotted showing the relationship between molecular weight and K_d. This is diagrammatically represented in Graph 5. The curve represents the working range of the Gel.

If now a saliva sample is run on this column and the fractions tested for/



Plot of the K_d volume against logarithms of the molecular weight for the different proteins on Sephadex G200. Inserted figure shows the whole separation range: 1. Sucrose 2. Vitamin B_{12} 3. Cytochrome C 4. Alpha Chymotrypsin 5. Ovalbumin 6. Gamma globulin and 7. Blue dextran.

for plasminogen as described then V_e may be determined. From the formula above, K_d is calculated and by reference to the standard curve for G200, the corresponding molecular weight may be obtained.

In this experiment, the information required was whether or not those fractions that displayed activator activity on bovine fibrin plates after being combined with streptokinase contained protein whose molecular weight was in the same order of magnitude as that of plasminogen. If the fractions did coincide with such a molecular weight, then the probability of them containing plasminogen as distinct from some other streptokinase-activated proactivator would be extremely high.

Summary of Results: Table 72. (Appendix 1).

Fraction 1. Graph 6.

Fraction numbers 37 - 44 produced lysis on SBFP when combined 1:1 with streptokinase.

Molecular weight range : 95,000 - 44,000
Molecular weight of fraction which gave maximum lysis 58,880.

Fraction 2. Graph 7.

Fraction numbers 50 - 58 produced lysis as above.

Molecular weight range : 138,000 - 70,790.
Molecular weight of fraction which gave maximum lysis 117,500.

Fraction 3. Graph 8.

Fraction numbers 40 - 47 produced lysis as above.

Molecular weight range 102,300 - 53,700.
Molecular weight of fraction which gave maximum lysis 93,330.

Fraction 4. Graph 9.

Fraction numbers 35 - 42 produced lysis as above.

Molecular weight range 107,000 - 52,480.

Molecular/

Molecular weight of fraction which gave maximum lysis 52,480.

Fraction 5. Graph 10A.

Fraction numbers 43 - 46 produced lysis as above.

Molecular weight range 89,130 - 66,070.
Molecular weight of fraction which gave maximum lysis 74,130.

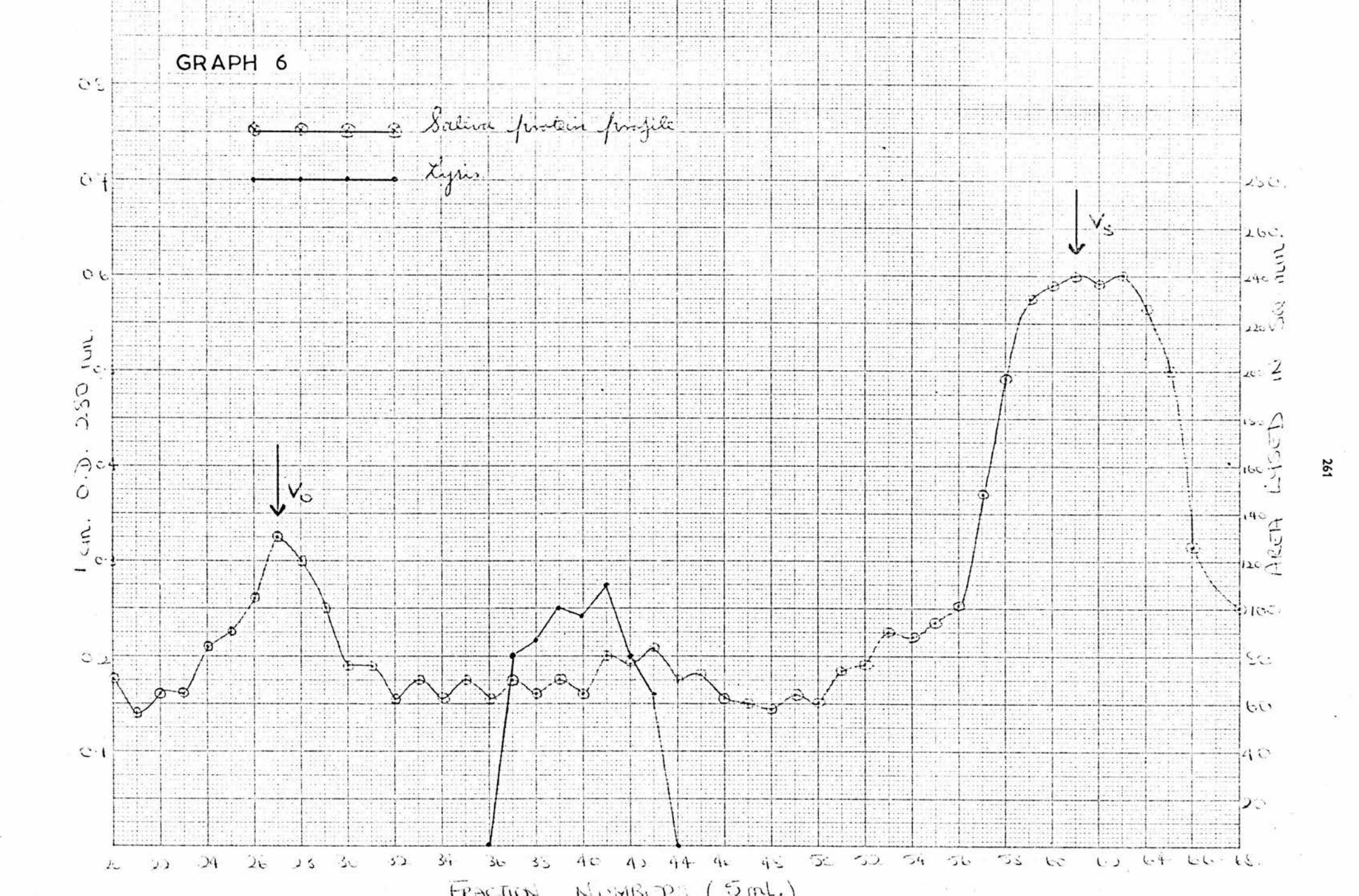
Fraction 6. Graph 11A.

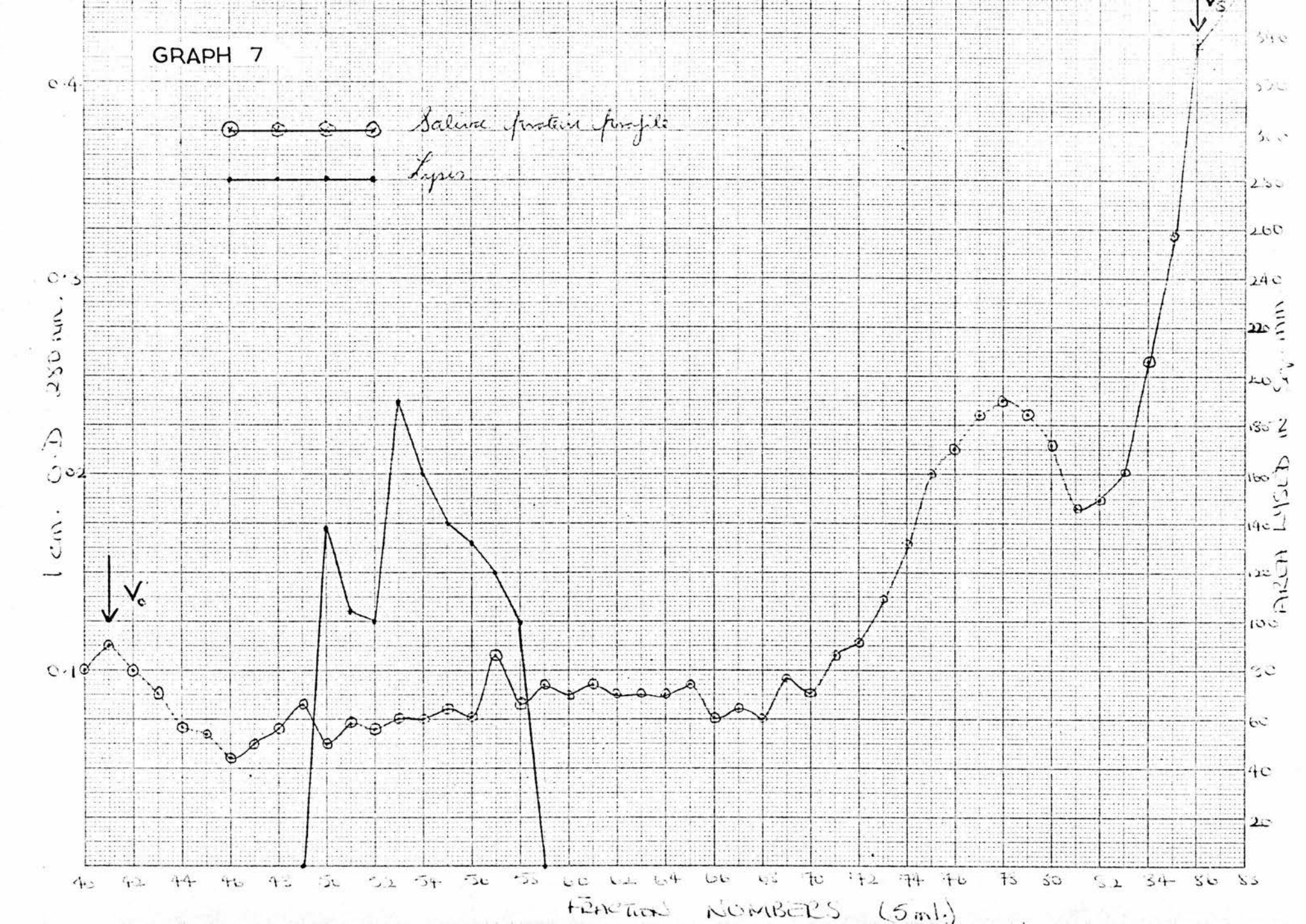
Fraction numbers 37 - 43 produced lysis as above.

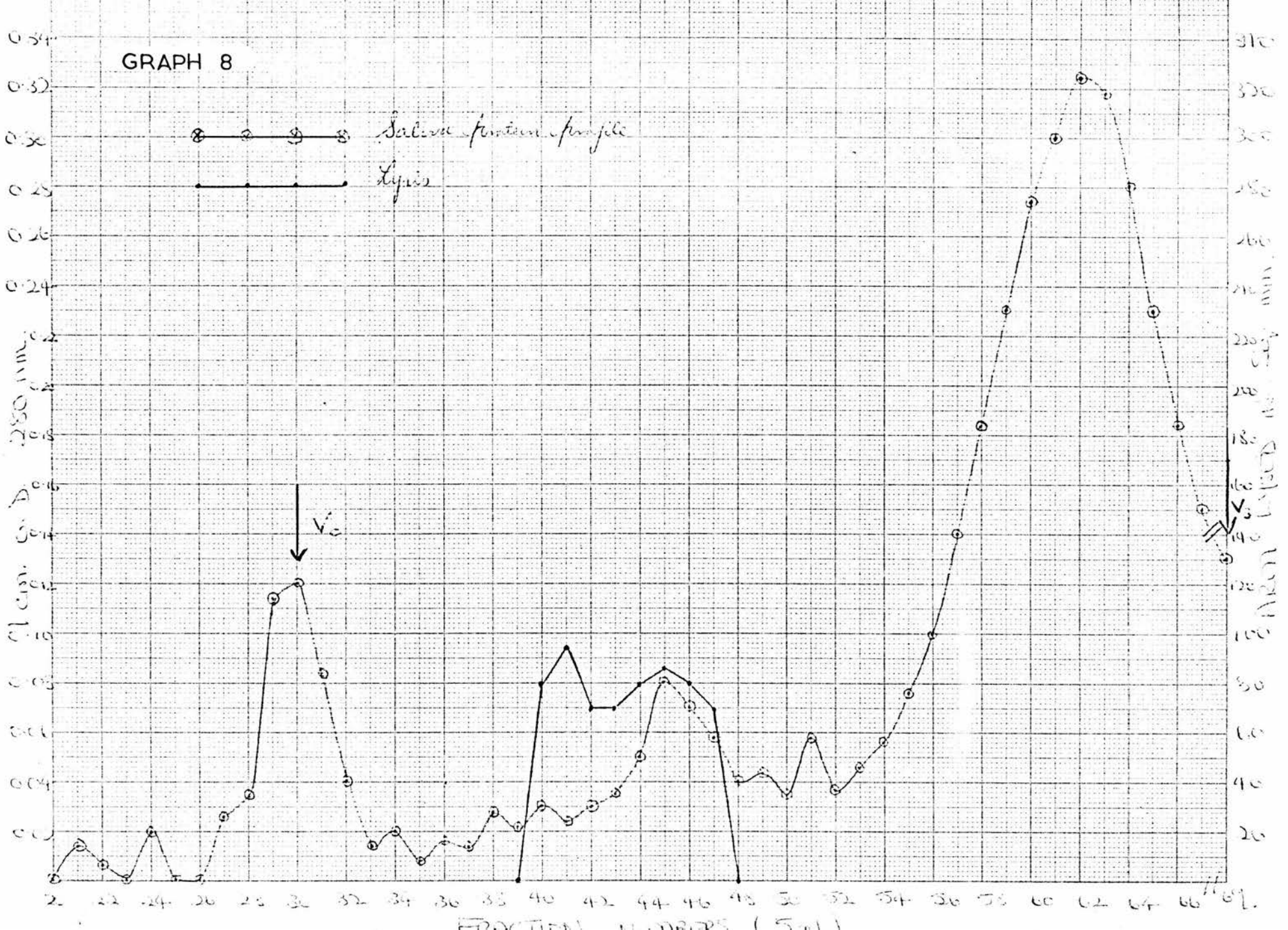
Molecular weight range 67,610 - 38,000.
Molecular weight of fraction which gave maximum lysis 63,000.

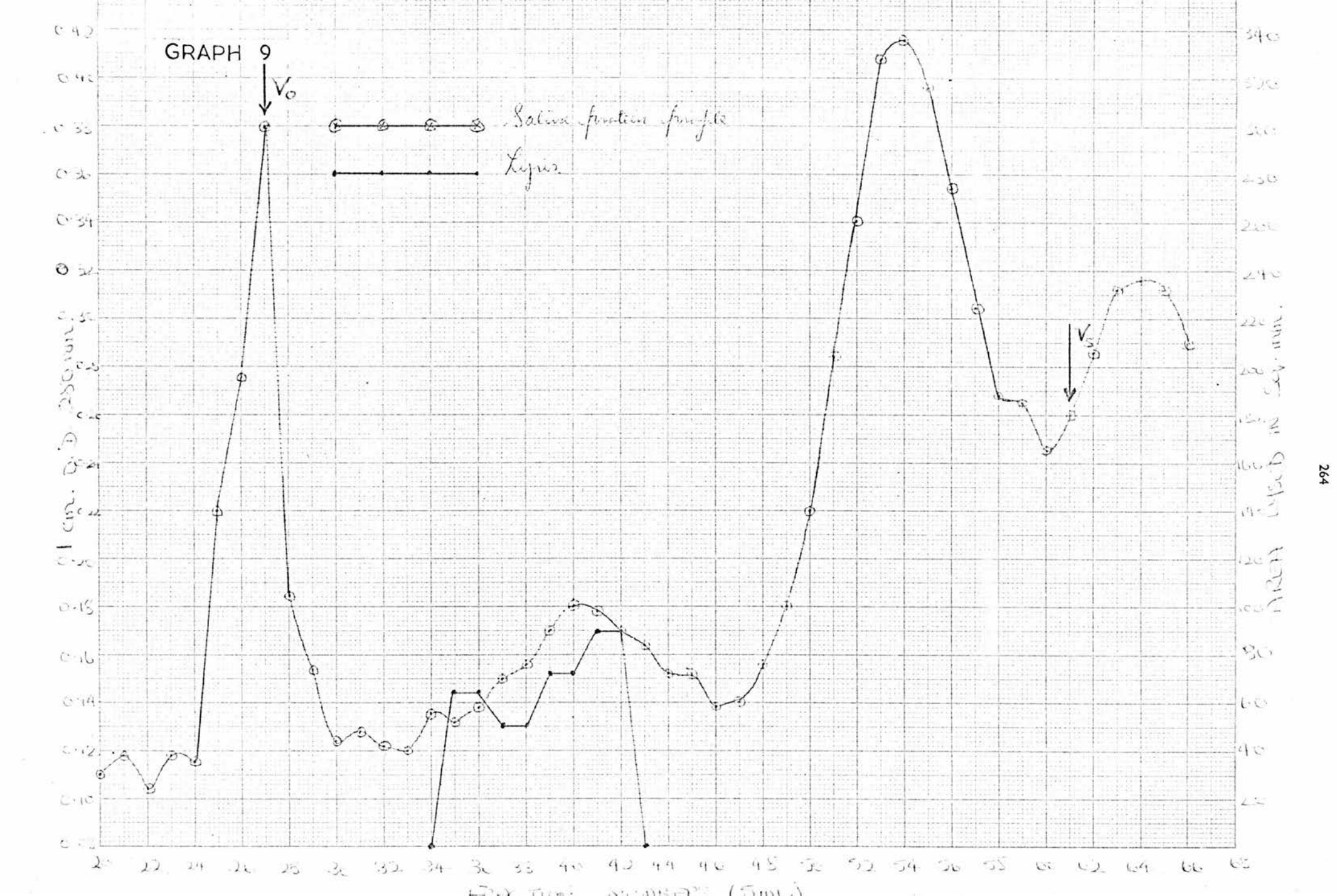
Duplicate graphs of Fractions 5 and 6 are illustrated displaying the additional information obtained by TRCHII (Graphs 10B and 11B).

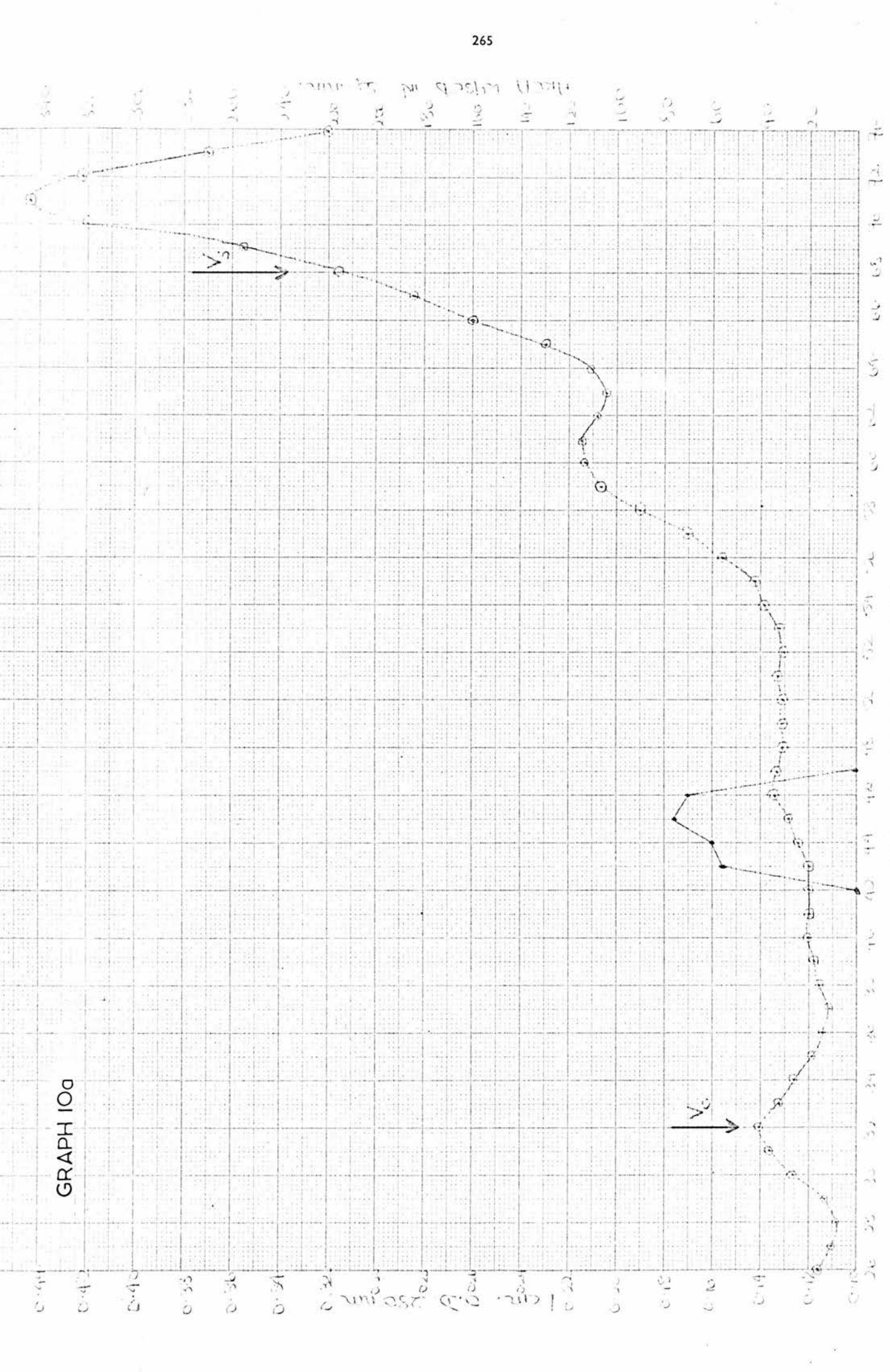
After the fractions had all been tested with streptokinase, the active fractions and a random selection of non-active fractions were subjected to TRCHII using the double adsorption technique. The results are described by the plots on these two graphs. Positive readings occurred only in those fractions which, when combined with streptokinase, produced lysis in SBFP. The shape of the lysis graph and the TRCHII graph are very similar and thus confirm beyond all reasonable doubt that the streptokinase-activated proactivator contained in those fractions with a molecular weight consistent with that of plasminogen was indeed, plasminogen.


GRAPH 6
Saliva protein profile
Xylis
V_0
V_S
1 cm. O.D. 280 nm.
AREA (cm²)
FRACTION NUMBERS (5 ml.)

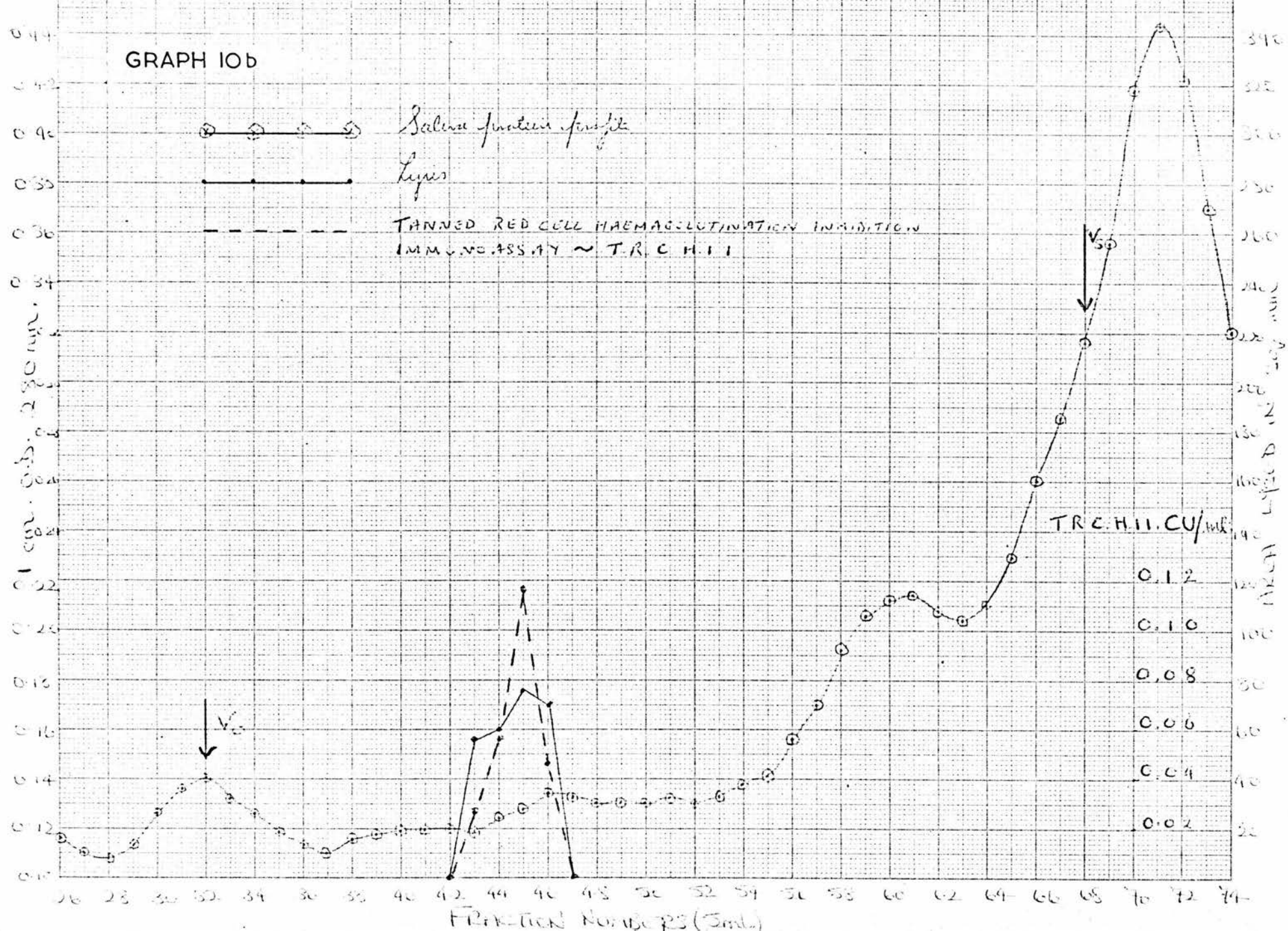

GRAPH 7
Saline fraction profile
Lipase
1 cm. O.D. 280 mμ.
0·4
0·3
0·2
0·1
V_o
V_e
FRACTION NUMBERS (5 ml.)
40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88

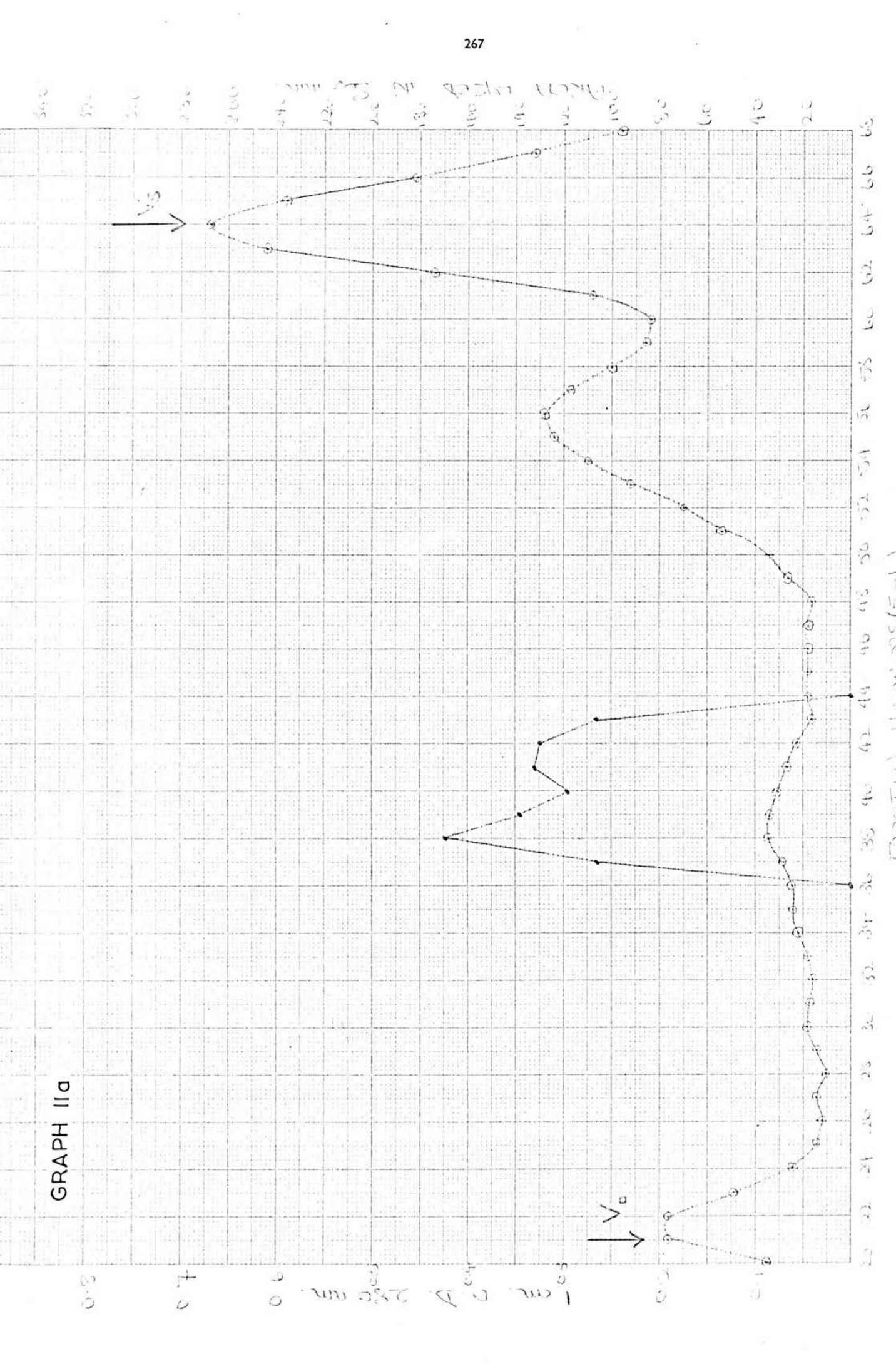

GRAPH 8
Sulcus fundus profile
Lipids
Vc
V3

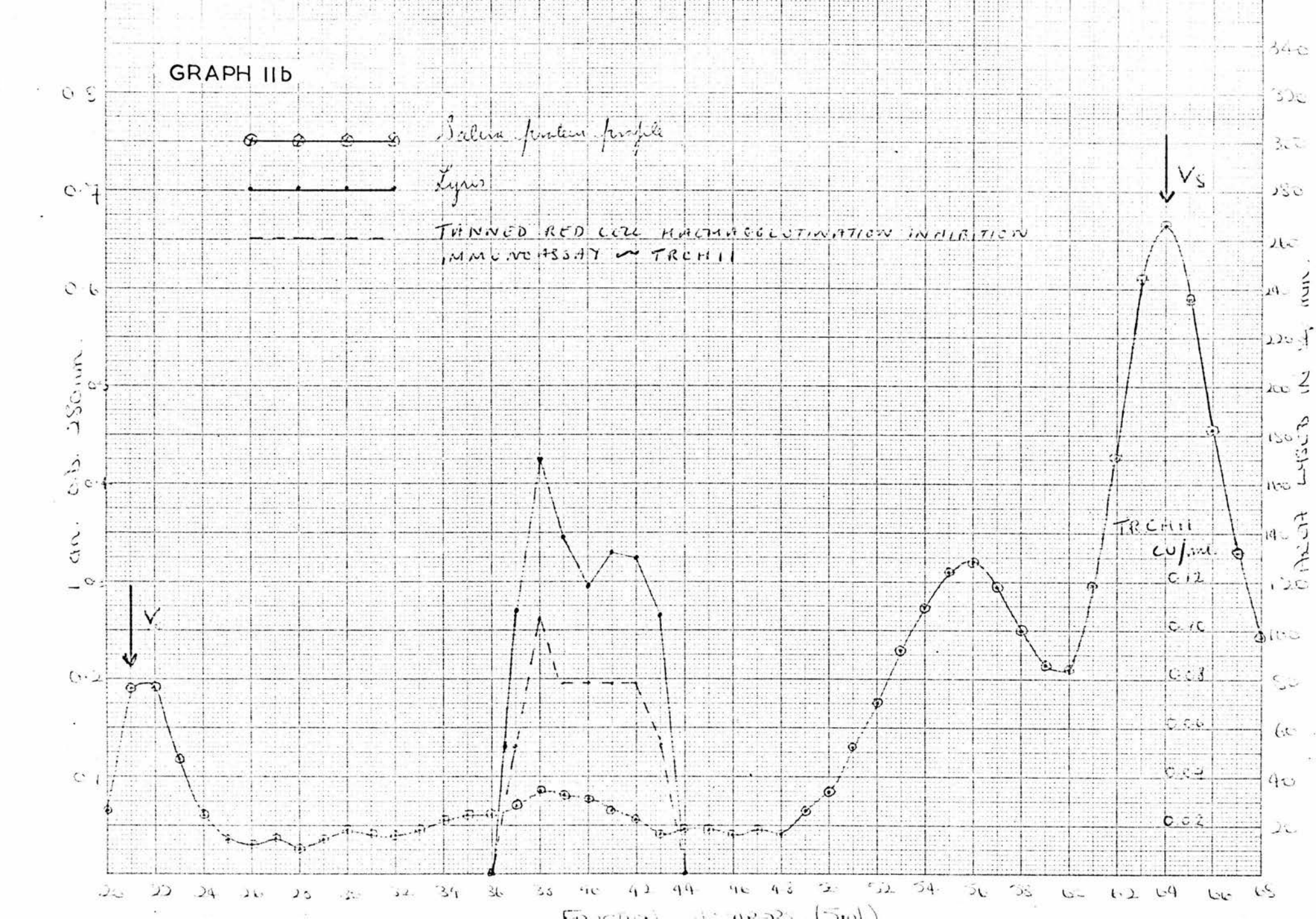

GRAPH 9
V_o
Saliva fraction fresh
Lyre
V_{30}



GRAPH 10a


GRAPH 10b
Saliva fraction profile
Lysis
TANNED RED CELL HAEMAGGLUTINATION INHIBITION IMMUNOASSAY ~ T.R.C H.11
V_0
V_{50}
TRC.H11.CU/ml
0.12
0.10
0.08
0.06
0.04
0.02
FRACTION NUMBERS (5ml.)


GRAPH IIa


GRAPH IIb
Saliva fraction profile
Lysis
TWINNED RED CELL HAEMAGGLUTINATION INHIBITION IMMUNOASSAY ~ TRCHII
V
Vs
TRCHII
CU/ml.
0.12
0.10
0.08
0.06
0.04
0.02
1 cm. abs. 350nm.
0.9
0.7
0.6
0.5
0.4
0.3
0.2
0.1
340
300
260
220
200
180
160
140
120
100
80
60
40
20
268

CHAPTER 3

Experiment.

Object: To examine the streptokinase-activated proactivator activity of the cellular components of MNS using SBFP and fibrinolytic autography.

Materials:

1. Salivary pellet. Stimulated MNS (Methodology, p. XXVI) was collected from 20 healthy persons with no clinical evidence of gingivitis. Each MNS sample was centrifuged at 4°C, 4154 g. for 20 minutes after which the supernatant was discarded and the pellet washed in tris buffer by centrifugation 3 times and finally resuspended in 0.5 ml. of tris. The washing was necessary to remove plasminogen in solution, but it also removed many epithelial cell fragments, leukocytes and bacteria.

2. Oral bacteria. The same isolates, suspensions and filtrates were used as were prepared in Part IV, Chapter 4, Experiment 2.

3. Leukocytes. The same suspensions of washed leukocytes in Heparin-Saline-Dextrose (HSD), MNS supernatant and freeze-thawed cells in MSD as in Part IV, Chapter 5, Experiment 1.

4. Oral epithelial cells. Buccal and tongue scrapings were made with a wooden tongue spatula. The scrapings were pooled to form dense concentrations of buccal and tongue cells. The two sets of cells were then washed by centrifugation in tris buffer and finally resuspended and doubly diluted to form several concentrations of cell suspensions. A cell count was carried out upon each prepared sample using an Improved Neubauer counting chamber.

5. SBFP. (Methodology, p. IV).

6. Tris buffer, pH 7.8, 0.15M. Negative control.

7./

7. Urokinase, 5 Ploug units/ml. pH 7.8, 0.15M. Positive control.
8. Streptokinase. 2,500 units/ml. (SK).
9. Molar Epsilon-amino-caproic acid (eACA).
10. Bovine fibrin autography.

Methods:

1. SBFP

Each of the cell preparations was divided into two. One half was mixed 1:1 (v/v) with tris buffer while the other half was similarly mixed with streptokinase. Each preparation was plated (3 x 0.03 ml.) upon SBFP and upon SBFP containing 0.1 ml. of molar eACA. The plates were incubated at 37°C for 19 hours and controls were set up as described in Part II, Experiment 3, Fig. 3. In addition, streptokinase was set up as a negative control.

2. Bovine fibrin autography.

.01 ml. of the cell suspensions were incorporated within the fibrin films (Methodology, p. V).

Results:

1. SBFP.

Salivary pellet. All 20 specimens lysed SBFP when mixed with SK (Mean 118 $\pm$ 44 sq. mm.) There was no lysis in the absence of SK nor in the presence of eACA.

Bacteria. Of all the bacterial preparations, only the BHS suspension caused lysis and BHS filtrate probable lysis of SBFP alone. No activity was recorded on SBFP with eACA by any preparation. The addition of SK to concentrated preparations brought about lysis with BHS, bacterial/

bacterial sweep and with the mixed bacterial suspension and probable lysis with bacterial isolates and BHS filtrate. When eACA was present in the plates, however, no lysis occurred and probable lysis was present only with the isolates and the bacterial sweep.

Leukocytes. Upon SBFP alone, the greatest activity was brought about by freeze thawed cells followed by those resuspended in MNS supernatant and very little activity from cells that had been washed in saline, and then only at high concentration. The addition of SK increased lysis considerably in all cell preparations, but the same pattern of relative activity existed. In the absence of SK and upon SBFP containing eACA only very concentrated suspensions of freeze-thawed cells caused any lysis. Upon the same plates but with SK present in the suspension, activity reappeared in the most concentrated samples, in the same order of relative activity as before, but reduced compared to the SBFP without eACA.

Oral epithelial cells. The buccal and tongue epithelial cell preparations did not lyse SBFP. Lysis was produced on SBFP when SK was included with the cells, and this lysis did not occur if eACA was also present.

2. Bovine fibrin autographs.

Salivary pellet. Isolated foci of lysis were related to epithelial cells in the presence of SK. (Fig. 59). No lysis occurred in the absence of SK or in the presence of eACA.

Bacteria. Foci of lysis, often coalescing to lyse the whole film, were seen in the presence of SK (Fig. 60). No lysis occurred in the absence of SK or in the presence of eACA.

Leukocytes. Foci of lysis were related to leukocytes especially where they were present in clumps but only in the presence of SK. (Fig. 61 and/

and 62). No lysis was present in the absence of SK or in the presence of eACA.

N. B. No lysis was seen in relation to erythrocytes also present in the leukocyte suspensions either with or without SK.

Epithelial cells. Foci of lysis were related to both nucleated and anucleated epithelial cells from the cheek (Fig. 63 and 64) and the dorsum of the tongue (Fig. 65) in the presence of SK but once more, no lysis occurred in the absence of SK or in the presence of eACA.

Discussion:

Using fibrinolytic autography and SBFP, the cellular components of MNS have been shown to have a streptokinase-activated proactivator activity which was inhibited with eACA. The study of the individual cellular components complemented the findings of the whole salivary pellet. On the fibrin autographs, bacterial morphology was altered with prolonged incubation with SK and extreme lysis (Fig. 66 and 67). The possibility arises that this might be related to the streptodornase content of the commercially available SK (Varidase). As purified SK was not available, this possibility was not investigated further. The significance of this observation is that the reaction appeared to be non-specific in relation to bacteria (i. e. all isolates were affected) and this type of activity must be considered when any test solution that is contaminated is examined for proactivator activity with SK. Human epidermis has a SK-activated proactivator activity (Nishioka and Ryan, 1971) and it has been shown that oral epithelial cells have a similar property. Troll and Sherry (1955) suggest two possibilities to explain interaction with SK and proactivator: SK may remove an inhibitor, or it may itself combine with the proactivator to form the activator. Furthermore, it is postulated that plasminogen activator and inhibitor exist as an easily dissociable complex in the blood (Wolf, 1968). It is, therefore, possible that in the epidermis (Nishioka and Ryan, 1971) the inhibitor and the proactivator of fibrinolysis may/

Fig. 59.

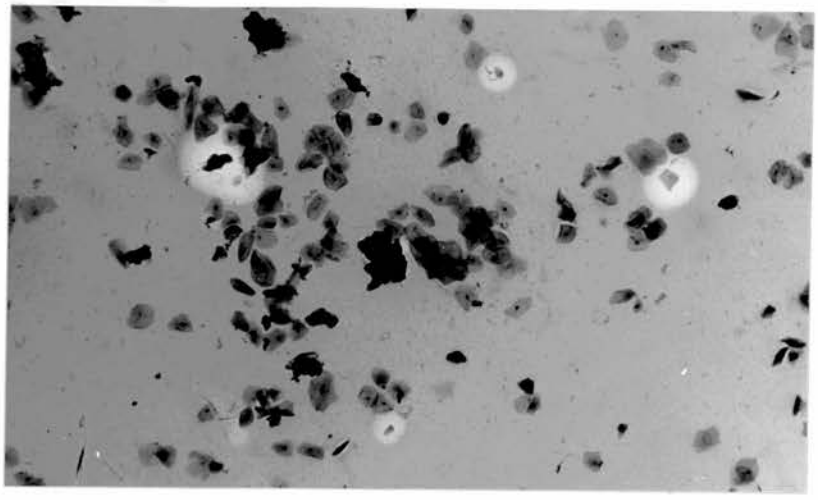


Fig. 59.

Fibrinolytic autograph of washed salivary pellet with streptokinase included in the bovine fibrin film and showing foci of lysis related to isolated cells. Incubated for 90 minutes at 37°C. Mag. x 48.

Fig. 60.

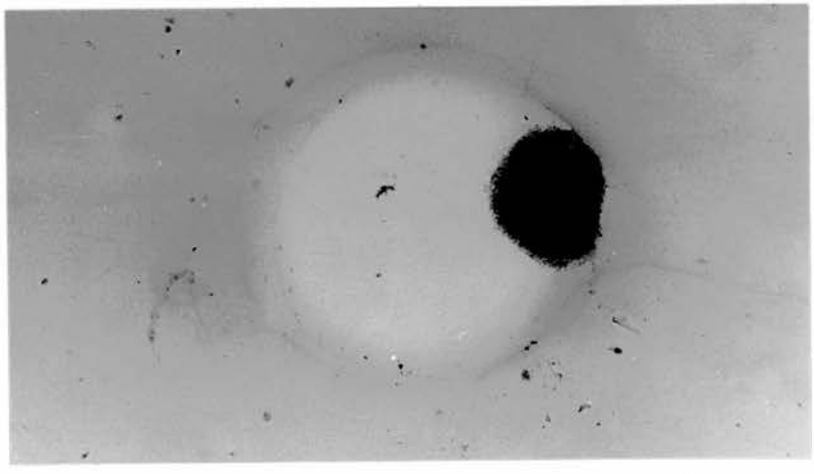


Fig. 60.

Fibrinolytic autograph of Alpha Haemolytic Streptococci isolated from MNS with streptokinase included in the bovine fibrin film. Lysis related to a clump of bacteria. N. B. The bacteria have fallen to one side of the focus of lysis during incubation due to gravity. Incubated overnight at room temperature and 1 hour at 37°C. Mag. x 48.

Fig. 61.

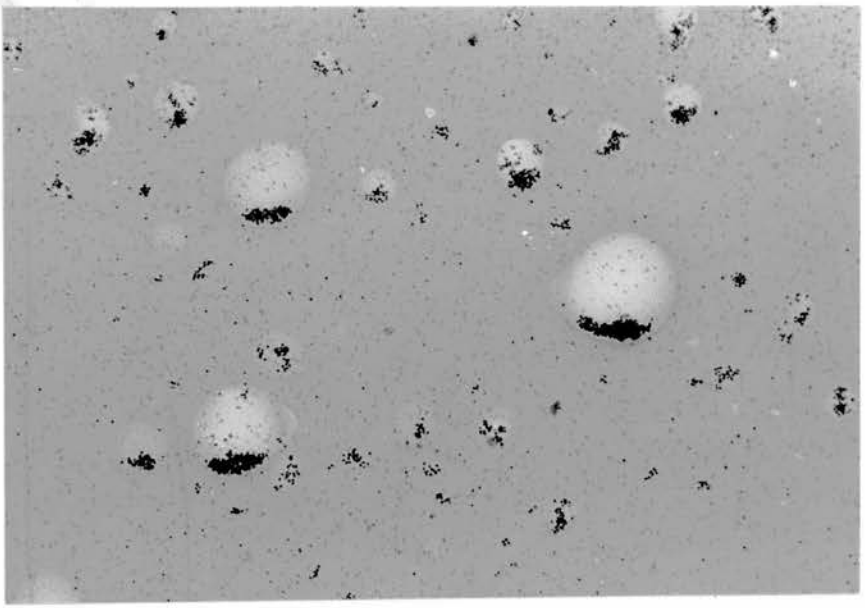
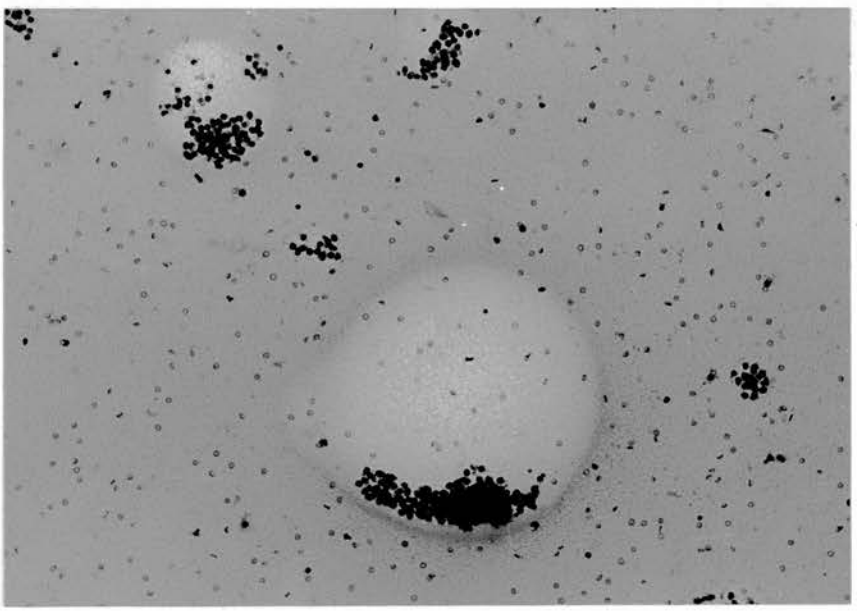


Fig. 62.



Figs. 61 & 62.

Fibrinolytic autograph of fresh leukocytes suspended in saline and included together with streptokinase in bovine fibrin showing lysis related to clumps of leukocytes but not to the erythrocytes which were also present. N. B. The leukocytes have fallen to one side of the foci of lysis during incubation due to gravity.

Incubated overnight at room t emperature and 1 hour at 37°C.
Mag. Fig. 61 x 48. Fig. 62 x 120.

Fig. 63.

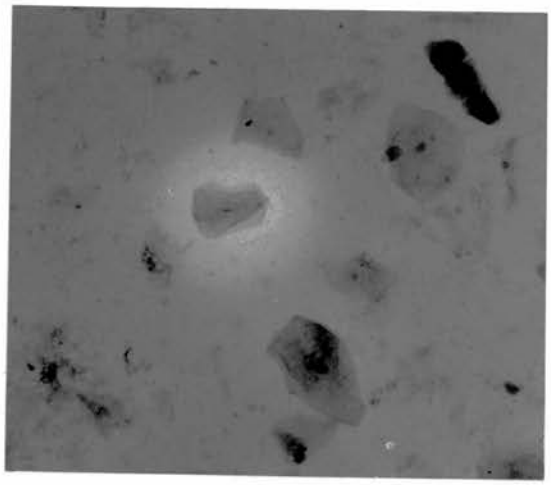
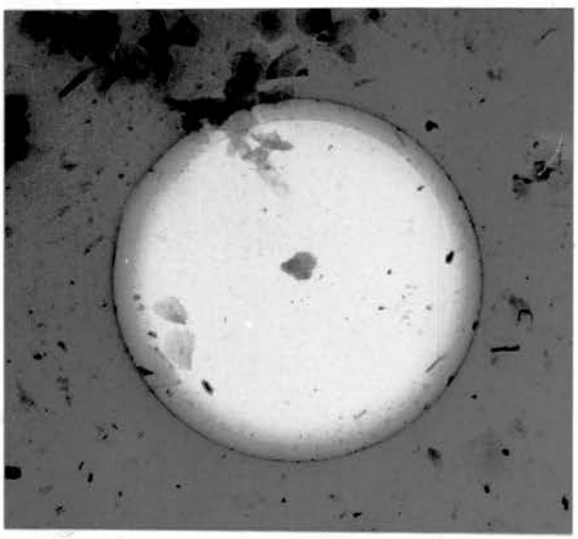


Fig. 64.



Figs. 63 & 64. Fibrinolytic autographs of a suspension of buccal cells in tris buffer included together with streptokinase in bovine fibrin , showing lysis related to nucleated and anuclear cells. Incubated overnight at room temperature. Mag. Fig. 63 x 120 Fig. 64 x 48.

Fig. 65.

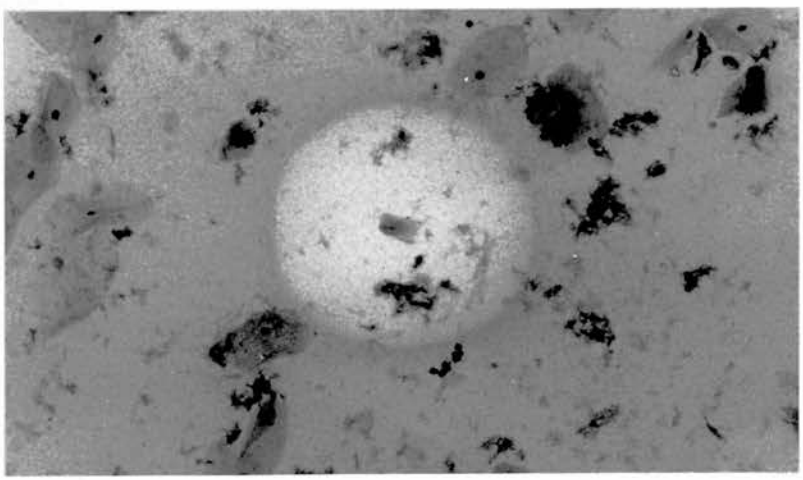


Fig. 65. Fibrinolytic autograph of a suspension of dorsum tongue cells in tris buffer together with streptokinase in bovine fibrin , showing lysis related to an anuclear cell fragment. Incubated for 1 hour at $37^{\circ}C$. Mag. x 120.

Fig. 66.

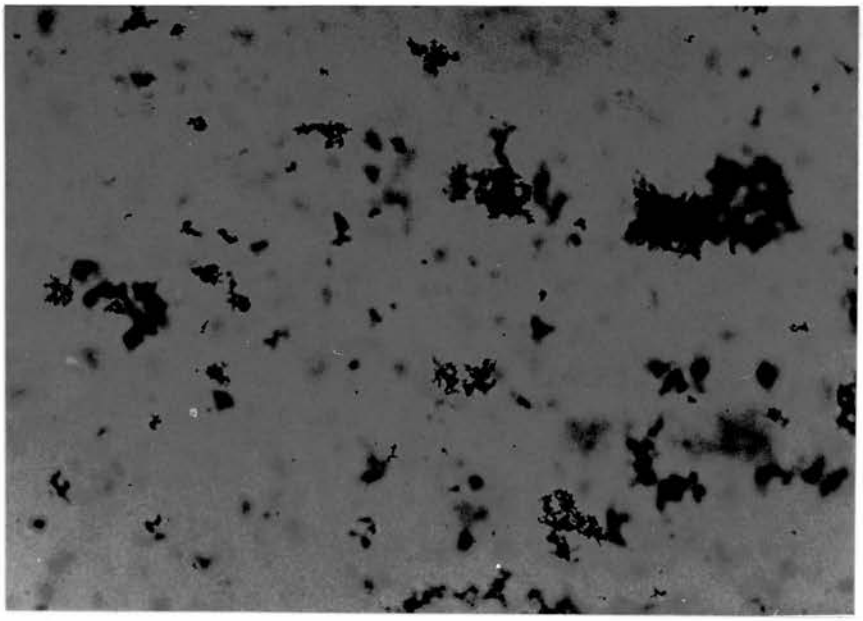


Fig. 66.

Fibrinolytic autograph of Alpha Haemolytic Streptococci isolated from MNS showing no lysis with bovine fibrin. Incubated overnight at room temperature. Mag. x 300.

Fig. 67.

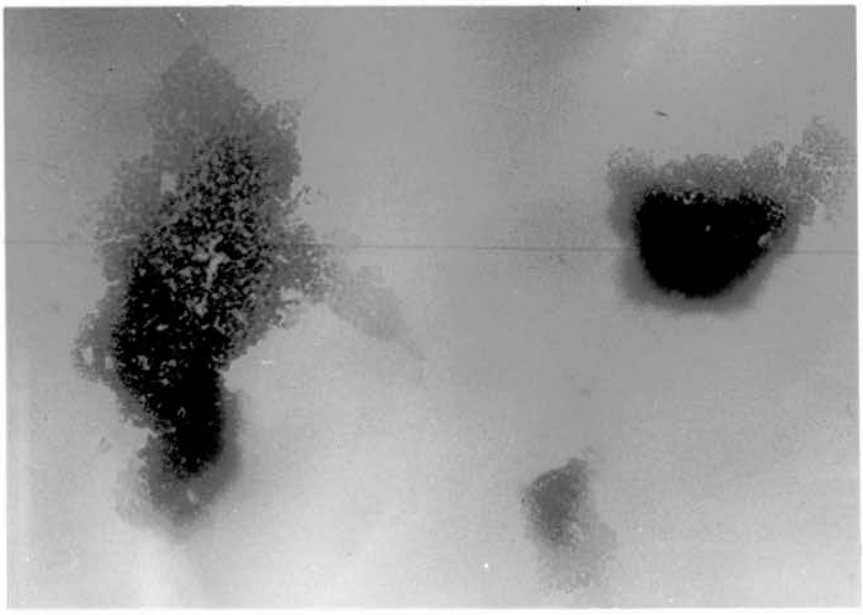


Fig. 67.

Fibrinolytic autograph similar to Fig. 66 except that streptokinase was included in the bovine fibrin film. Total lysis of the fibrin has occurred with gross alteration of bacterial morphology. Mag. x 300.

may exist as a complex which is dissociated by Sk-treatment. This might apply also to oral epithelial cells. This Thesis is concerned with the source of the SK-activated proactivator activity and the nature of that activity will constitute further work. However, a preliminary indirect immunofluorescence study of human buccal epithelial cells using antiplasminogen has already suggested that the buccal cell associated activity is due to plasminogen (Fig. 68). It is not known whether this plasminogen is produced from within the cell or adsorbed onto its surface from saliva. Recent permeability studies of the oral mucosa (Hopps and Squier, 1976) suggest that a molecule as large as plasminogen would not diffuse through the oral mucosa.

Conclusion:

1. All the cellular components of MNS have SK activated pro-activator activity when studied in isolation.

2. In view of the fact that some individual cells showed activity with fibrinolytic autography and with due regard to their size and number, epithelial cells are the most important cellular proactivators in MNS.

Fig. 68.

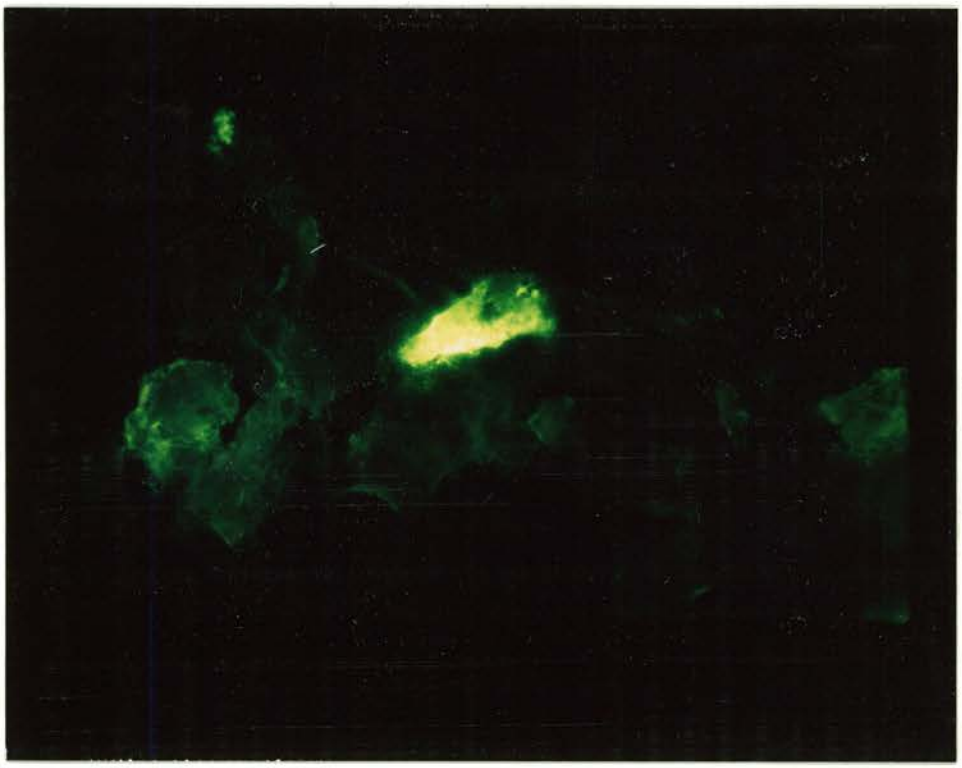


Fig. 68.

Indirect immunofluorescence using antiplasminogen serum showing strong fluorescence of a single cell and slight fluorescence of many cells in a buccal smear.

CHAPTER 4.

Discussion and Conclusions:

From these experiments, it can be concluded that there are two components in MNS reacting with streptokinase. One of these is in solution and the other cell associated. The two components have been separated and it has been demonstrated that a soluble streptokinase activated proactivator is present in MNS, parotid and submandibular/sublingual saliva. This proactivator activity remains when all activator activity has been removed either by filtration or centrifugation.

The nature of this soluble proactivator has been investigated by observing its activity after combination with streptokinase upon S BFP, by studying it in an immunodiffusion system and by TRCHII, and lastly by gel chromatography and observing at what molecular weight plasminogen-like activity occurred. The results were consistent qualitatively and all the evidence indicated that this streptokinase-activated proactivator was plasminogen.

The major source undoubtedly appeared to be the paired salivary glands but a little plasminogen may re-enter the MNS from the mucous membrane to which some may be adsorbed (or produced) for the high speed supernatant MNS plasminogen level as indicated by SK-activated proactivator activity upon SBFP was consistently higher than that of isolated parotid or submandibular/sublingual saliva albeit the difference was small.

Washed salivary pellet cells capable of producing only a little lysis upon SHFP when placed in a solution of plasminogen of 0.05 CU/ml. produced lysis in the same order of magnitude as achieved by placing the washed cells back in the MNS supernatant. Two points arise from this. Firstly, the plasminogen solution, based upon the calculated amount of plasminogen in MNS, behaved very like MNS supernatant and secondly, the activator activity of the washed cells, and these must/

must necessarily be mainly epithelial cells, can activate the plasminogen in solution to bring about lysis. This then suggests that the fibrinolytic activity in MNS is generated by plasminogen in solution and a cell-associated activator.

The findings of Part IV indicate that although salivary leukocytes may have a small activator activity, both their contribution and that of the oral bacteria is negligible and the predominant activator cell is the desquamated epithelial cell.

It is concluded then from this series of experiments that the soluble proactivator in MNS, parotid and submandibular/sublingual saliva is plasminogen and that in vivo, the plasminogen activator is cell-associated and does not appear to diffuse into solution. Hence, plasminogen in MNS is not immediately converted into plasmin but is so converted if the activator cell (epithelial cell) and the MNS are incubated together, e.g. upon a SHFP.

Quantitatively, the results are less satisfying. The level of plasminogen present in the average MNS from clean dentate and edentate mouths appeared to be in the region of 0.05 CU/ml. The control experiments indicated a considerable loss, in the region of 50% and clearly further work needs to be done to improve this. An attempt to produce a satisfactory euglobulin is one approach; immunoelectrophoresis another. The writer believes, however, that the correct order of magnitude has been established.

PART VI

CONCLUSIONS

PART VI

Chapter 1

Summary of Results and Conclusions

Part I concludes with a statement of Objectives, viz.

1. Does human MNS possess specific plasminogen activator activity?
2. If activator activity is present, what is its source?
3. What is the nature of 'proactivator' in saliva?

Question 1

It was demonstrated, consistently, that MNS had plasminogen activator activity and that within the sensitivity of the fibrin plate technique, this activity appeared to be specific as no lysis took place in the presence of eACA, a specific competitive inhibitor of plasminogen activation.

Conclusion: MNS possesses specific plasminogen activator activity.

Question 2

When MNS was rendered cell free either by filtration or centrifugation the activator activity (even in concentrated supernatant) was lost. Isolated parotid and submandibular/sublingual secretions occasionally showed activity, but when contaminating cells were removed, the secretions became inactive. The concentrated supernatant of pooled MNS was examined by gel chromatography and each fraction was tested for fibrinolytic activity. No fraction lysed an SHFP even after concentrating 25 times. Salivary pellet separated on a density gradient showed that although part of the activity was confined to the least dense fraction, this was related to cellular material and no soluble activator was present. From these results, it is deduced that there is no soluble plasminogen activator in MNS and that the activator activity is cell associated. /

associated. Experiments have been described and discussed which systematically examined the cellular components of MNS and which demonstrated that it was epithelial cells and epithelial cell fragments which had activator activity.

Conclusion: There is no soluble plasminogen activator in MNS. The activator activity is associated with epithelial cells and cell fragments.

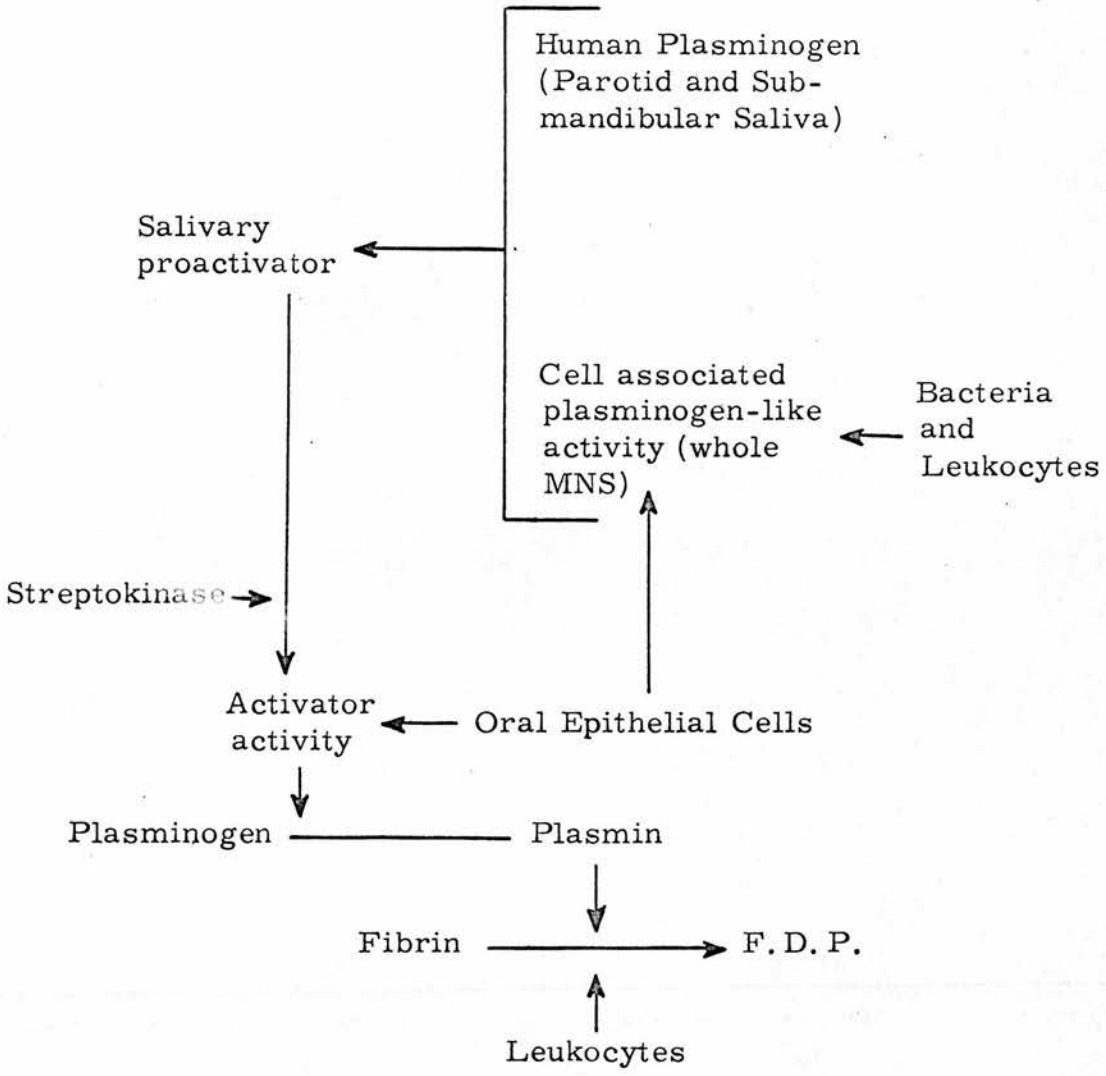
Question 3

Streptokinase-activated 'proactivator' activity has been demonstrated both in MNS and in the supernatant of MNS. The activity in MNS however was much greater and was separated into two compartments, one in solution, the other cell associated. Using the techniques of immunodiffusion, TRCHII and gel chromatography, plasminogen has been established as being present in MNS, parotid and submandibular/sublingual saliva and in sufficient quantity as to account for all the streptokinase-activated proactivator activity in solution. No experiments however were carried out to investigate the presence of any streptokinase-activated proactivator that was not plasminogen. Systematic examination of the individual cellular components of MNS showed activator activity with streptokinase to be associated with all cell types, but in view of frequent individual cell activity, their numbers and size, the epithelial cells were the most important. With both bacteria and leukocytes much higher numbers than found in saliva were required to produce measurable lysis. The nature of this cell associated proactivator activity has been discussed.

Conclusion: Proactivator exists in MNS in two compartments: soluble and cell associated. The evidence shows that the soluble proactivator is mostly, if not entirely, plasminogen.

The cell associated proactivator activity is a property of all cell types present but is predominantly associated with desquamated epithelial cells.

To summarise the major plasminogen activating system in human MNS may be diagrammatically represented.



PLASMINOGEN ACTIVATOR SYSTEM IN
HUMAN MIXED NATIVE SALIVA

CHAPTER 2

COMMENTS

Function

The question of "function" inevitably arises whenever an enzyme system is studied. It has been suggested that urokinase may serve to maintain the patency of the ureter (McNicol et al, 1961) but no similar function can be attributed to saliva in respect of the parotid and submandibular ducts for here the activator is not in solution.

1. Inevitable Action.

In this study as well as in the investigations on human vaginal epithelium (Tympanidis et al, 1968), rat vaginal epithelium (Henrichsen and Astrup, 1967) and human oral epithelial cells (Birn and Fejerskov, 1971) it was found that a minority of the epithelial cells were fibrinolytically active and the activity was not restricted to any specific cell layer. It is not at all clear what the value of fibrinolytic activity in these sites could be, but Wunschmann-Henderson and Astrup (1972) suggest that fibrinolysis may be involved in the process of cell desquamation. This is, at least, consistent with the presence of a few, about 2%, fibrinolytically active desquamated epithelial cells in MNS. Plasminogen in the parotid and submandibular/sublingual secretions has been established and it may be present as a filtrate of blood. A future experiment is intended to establish whether or not a relationship exists between plasminogen levels in plasma and MNS. It has been shown that the supernatant of MNS is fibrinolytically inactive, but when incubated with washed desquamated epithelial cells derived from salivary pellet, the suspension produced lysis upon an SHFP. The same result was obtained by incubating the cells with a concentration of plasminogen calculated to be equivalent to that present in MNS. The cells alone produced only/

only a little activity. Thus it may be that it is the method which, as it were, 'confers' fibrinolytic properties upon MNS by incubating together a weakactivator and plasminogen. In vivo with a clearance time for MNS of twenty minutes, the fibrinolytic activity may effectively not exist. It is a well established practice in dentistry to try and keep saliva out of the socket immediately following extraction of a tooth for it has been observed that when saliva has contaminated the wound prior to a clot forming, then the clot frequently lyses rapidly and the wound becomes infected. This is generally attributed to infection by the oral flora and hence the pre-extraction cleansing of the mouth when ever possible. Given the vast number of oral bacteria, the depressingly large number of teeth extracted daily and the relatively infrequent occurrence of 'dry sockets', it is possible that the bacteria are not responsible. The mouth cleansing procedures will greatly reduce the numbers of desquamated epithelial cells in MNS and hence the number available to be incorporated in a blood clot within a socket. Desquamated epithelial cells incubated within the blood clot in vivo may greatly reduce the clot lysis time. This suggests future experimental work and as a preliminary, the writer and his colleagues have incorporated urokinase in rat tooth sockets with tris buffer being incorporated in control sockets. Where urokinase has been incorporated, dry sockets have developed.

Thus the fibrinolytic activity of MNS may be simply an inevitable consequence of incubating desquamated epithelial cells with plasminogen and the danger of allowing saliva into a socket may not be related to bacteria or the fibrinolytic activity of MNS, per se, but rather to desquamated epithelial cells that become incorporated in the blood clot.

2. Evolutionary advantage.

Nour-Eldin and Wilkinson (1957) list the many blood clotting factors present in MNS and observe that saliva mixed with blood brings about a clotting time of between 2.5 - 4 minutes even when the saliva is derived/

derived from a haemophiliac and mixed with a haemophiliac's own blood. Mason and Chisholm (1975) list the many antibacterial substances present in saliva. If these are considered with the mechanical cleansing effects of saliva, then there is a sound basis for the idiom "to lick one's wounds". It would confer little advantage if a potent fibrinolytic substance was also in saliva at the very time when a stable clot was required. Therefore, the absence of a soluble plasminogen activator in MNS may be of some advantage.

3. Comparative Physiology.

With the exclusion of a soluble plasminogen activator in MNS, there is no need to attribute any atavistic function to salivary fibrinolysis but the species specific plasminogen activator in the saliva of the common vampire bat, Desmodus rotundus (Hawkey, 1966) and the equally specific (but for different species) plasminogen activator in the saliva of Disemus youngi (Cartwright and Hawkey, 1968) illustrate a vital role for salivary fibrinolysis in some animals.

Future studies.

The work carried out for this Thesis has raised more questions than it has answered.

1. Plasminogen. A more accurate quantitative technique for saliva requires to be developed. A possibility is the production of a euglobulin fraction obtained by dialysis and ammonium sulphate precipitation of parotid and submandibular/sublingual secretions.

2. Physiological studies concerning plasminogen levels in secreted salivas with respect to levels in plasma. The effect of age, sex, menstruation etc. might be studied.

3. The activator has been described as 'cell associated' but from where in the cell does it arise? The fragments and anuc-

lear/

lear cells suggest it may be cell bound and certainly in preliminary investigations by the writer, no 'release' has yet been obtained using sonication or potassium thiocyanate.

4. How stable is this cell activator at various temperatures and pH? The early preliminary experiments which studied the stability of activator in MNS suggest it is not very stable at 37^{o}C, but systematic studies upon cells has yet to be carried out.

5. Are there any fibrinolytic inhibitors in MNS?

6. What is the relationship, if any, between the presence of plasminogen in MNS and the coagulant factors also present?

7. Is there an association between plasminogen levels in saliva and the finding that some patients bleed for an excessively long time after extraction of teeth and yet appear to fall within normal parameters when examined for a bleeding disorder and do not bleed excessively from a wound anywhere else in the body?

8. Is the plasminogen activating activity of some desquamated epithelial cells responsible for 'dry sockets'? If so, can preventive treatment be devised?

9. Spontaneous gingival haemorrhage is poorly understood. Could it be related to accumulated desquamated epithelial cells in the materia alba being allowed to incubate with plasminogen in the gingival fluid?

10. Is the clinical observation that with exception of gross traumatic wounds and clot filled sockets blood clots are not seen in the mouth (even after quite severe denture irritation associated with atrophy, ulceration and granulation tissue) attributable to the plasminogen activating activity of oral epithelial cells?

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